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SECTION MEETINGS

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Adrenal Repair and Chromatophore Expanding Activities of Corticotropin-B.* (20118)

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Recent reports have presented evidence for the possible existence of separate adrenocorticotropic factors concerned with adrenal ascorbic acid depletion (A.A.F) and with adrenal weight (A.W.F.)(1-4). However, the routes of administration, number of injections, the time required for the hormone to act, and the nature of the response elicited are different for the two types of assays. Therefore, such factors as rate of absorption and rate of inactivation within the animal body may affect the apparent A.W.F./A.A.F. ratio obtained for a given preparation. It is possible that preparations from different animal sources may be handled differently by the body. Contamination with other pituitary hormones may also influence the result; pituitary extracts rich in somatotropin are reported to give particularly high A.W.F./A.A.F. ratios(3). One group of investigators believes that A.A.F. and A.W.F. responses are due to a single substance(5).

A highly active product from pepsin digests of corticotropin has recently been isolated and designated as corticotropin-B(6). This product had an A.A.F. activity as high as 300 International Units per mg, and behaved as a pure substance in countercurrent distribution studies. It was of interest to determine the A.W.F. and chromatophore expanding activities of corticotropin-B in comparison with less highly purified materials having lower A.A.F. activity.

Materials and methods. The adrenocortico-

* The term "corticotropin-B" as used in this paper designates a product obtained by countercurrent distribution after fractionation of pepsin digests of corticotropin with oxycellulose. The designation "corticotropin-B" has been given to this substance because its properties are different from those of corticotropin(6), particularly with regard to its solubility behavior. Corticotropin-B shows no evidence of inhomogeneity by countercurrent distribution studies. Its method of preparation has been described(6).

TABLE I. Comparison of Adrenal Weight Activity of Preparations. A (alkaline extract of beef pituitaries) and E (corticotropin-B).

Preparation	Total amt inj., mg	No. of animals	Mean values: Body wt, g — Initial	Body wt, g — Final	Thymus, mg	Adrenal wt, mg ± S.E.
A	1.05	8	112	115	217	13.69 ± .68
A	15.00	3	120	129	—	19.03
E	1.05	10	108	114	127	18.68 ± .61
None	—	11	106	109	210	13.56 ± .58

tropic preparations used in this study were as follows: A, an alkaline extract of beef pituitary glands having an A.A.F. activity of less than 1 U/mg; B, Armour Acthar-A precipitated at pH 4.6, activity about 5 U/mg; C, Armour Acthar-A hydrolyzed for several hours in boiling 0.001 N HCl, and precipitated at pH 4.6, activity about 3 U/mg; D, partially purified corticotropin-B, activity about 175 U/mg; E, corticotropin-B, purified by counter-current distribution, activity about 250 U/mg. The animals used in this study were Sprague-Dawley male rats of 100 to 120 g body weight, hypophysectomized about a week prior to the test by the Hormone Assay Laboratories, Chicago, Ill. Injections of corticotropin in aqueous solution were administered subcutaneously.

Results. Exp. 1. In this experiment, the A.W.F. activity of preparations A and E were compared. Injections were continued for one week; the daily dose was divided into two injections except on Saturday and Sunday, when the usual daily amount was given in a single injection. The total number of injections was therefore 12. The rest of the details of the experiment are evident from inspection of Table I.

It is clear that 1.05 mg of preparation A produced no effect on adrenal size. 1.05 mg of corticotropin-B (preparation E) produced a moderate increase in adrenal weight which was highly significant ($P < 0.01$). The very small thymus glands found in the animals treated with preparation E provide further evidence of the adrenal-stimulating activity of this substance. The increment in adrenal weight in this group of animals was about the same as in those receiving 15 mg of preparation A. It does not necessarily follow, however, that preparation E is 15 times more potent in A.W.F. than is A, since this experiment gives us no information as to the dose-response relationship of various effective doses of either preparation.

Exp. 2. In this experiment, a more complete study was made, including dose-response curves for preparations B, C, and D. One hundred hypophysectomized rats were divided into 10 groups of 10 animals each, as shown in Table II. Injections were made twice daily over a 7-day period (total of 14 injections), and solutions were freshly prepared before each injection. The injection schedule was therefore slightly different from that of Exp. 1, so direct comparisons of activity of these preparations with those used in Exp. 1 would not be valid. The adrenal weight data in Table II may be plotted to yield straight lines with the following regression equations: B, $y = 19.31 + 9.03X$; C, $y = 16.42 + 3.84X$; D, $y = 24.60 + 9.03X$, where y = adrenal weight in mg, and X = log total dose. The amounts of hormones required to produce a 50% increase over control adrenal weight are calculated from these equations to be as follows: D 0.54 mg, B 2.09 mg, and C 32.00 mg. A direct comparison of potency on this basis

TABLE II. Dose-Response Data of Adrenal Weights in Hypophysectomized Rats Treated with Preparations B (pH 4.6 Precipitate of Armour Acthar-A), C (pH 4.6 Precipitate of Acid Hydrolysate of Armour Acthar-A), and D (Partially Purified Corticotropin-B).

Preparation	Total amt inj., mg	Body wt, g — Initial	Body wt, g — Final	Adrenal wt, mg
B	1.0	112	116	18.2
B	4.0	113	121	26.2
B	16.0	106	112	29.9
C	1.0	112	115	16.6
C	4.0	117	117	18.4
C	16.0	115	114	21.2
D	1.0	110	108	25.0
D	4.0	110	111	30.1
D	16.0	113	102	35.0
Controls	—	115	113	14.8

between preparation C and the other two preparations, however, must be subject to some reservations, since the data for C have a significantly different slope, and the highest dose of C gave somewhat less than 50% increment in adrenal weight; also, preparation D may be more potent in comparison to B than the data indicate, since the lowest dose of D gave more than 50% increase in adrenal weight.

Exp. 3. In addition to these observations on adrenal weight, a preliminary experiment was performed to determine whether corticotropin-B also possesses a higher degree of chromatophore-expanding activity than a cruder preparation. This was of interest in view of the recent claim that corticotropin and the chromatophorotropic hormone are identical(7,8), although this has been denied (9,10). We have tested the action of corticotropin-B on frog skin *in vitro*. Pieces of skin of *Rana pipiens* about 1.5 cm square were immersed in frog Ringer's solution containing hormone in concentrations of 0.01, 0.1, 1.0 and 10.0 μg/ml. Two hormone preparations were used: a relatively crude corticotropin acid hydrolysate (about 1.2 U per mg) and highly purified corticotropin-B.

There was a gradation in the degree of darkening produced. The skin exposed to 10.0 μg/ml of corticotropin-B was by far the darkest, while 1.0 μg/ml of corticotropin-B and 10.0 μg/ml of the cruder preparation produced moderate darkening of about equal degree. Concentrations of 0.1 μg/ml of corticotropin-B and 1 μg/ml of the cruder preparation produced slight darkening. No noticeable effect was produced by 0.1 μg/ml of the cruder preparation or by 0.01 μg/ml of either preparation. Corticotropin-B, therefore, appeared to have melanophorotropic activity on the order of 10 times that of the cruder corticotropin, but this ratio must be accepted with reservations, since the method used was at best no more than semi-quantitative.

Discussion. Activity in the adrenal ascorbic acid depletion test as high as 300 times that of Armour Standard LaIA ACTH has been reported for corticotropin-B(6). This is not necessarily the highest activity for corticotropin-B, since partial inactivation may occur in the process of working up the pure substance in the peak plates of the distribution. This partial loss of activity has been frequently observed, and is most marked in the most highly purified preparations. Corticotropin-B is obtained from pepsin digest of corticotropin derived from swine pituitary glands. The application of countercurrent distribution which produced through 450 transfers corticotropin-B, behaving as a pure substance, is a very critical criterion of purity. It is therefore of considerable interest that this substance not only possesses all three biological activities tested (adrenal ascorbic acid depletion, adrenal weight increase, and chromatophore expansion) but possesses them to a higher degree than do cruder preparations.

In the adrenal weight test, the dose-response curve for the acid-hydrolyzed material, preparation C, had a different slope than the curves for B or D. It is therefore possible that the nature of the A.W.F. is changed by the acid treatment. This change may be either in the way the active principle affects the adrenal, or in the way the body handles the injected material. Not only is it impossible to obtain a valid ratio of a single activity between two substances showing such a different slope of response, but comparison of A.W.F./A.A.F. ratios between such materials would be equally meaningless. Furthermore, the errors inherent in the assays make it difficult to determine such ratios with a practicable degree of precision. For these reasons, as well as because of the considerations outlined in the introductory paragraph, we have not attempted to calculate A.W.F./A.A.F. ratios for any of our preparations.

The method of preparation of corticotropin-B and the apparent high degree of purity of this substance(6) make it seem unlikely that the very high A.W.F. and chromatophorotropic activities are due to contaminants.

A reappraisal of certain controversial issues can be made in the light of these data on the hormonal activities of corticotropin-B; for example, are A.A.F. and A.W.F. activities due to the same or to different chemical entities? We now have an apparently pure substance exhibiting both activities, but this does not

preclude the possibility that some other substance present in ACTH-concentrates might have only one or the other activity. The characterization of one or more active substances other than corticotropin-B is required for satisfactory appraisal of the issue, and in some instances it might be necessary to prove the absence of corticotropin-B in certain concentrates before drawing conclusions about the presence of different hormonal entities in a given concentrate.

Corticotropin-B is from swine pituitaries. Any comparison of activities of various corticotropin preparations should take into account the species from which the glands were secured. The importance of this consideration has been established for certain other protein hormones. For example, vasopressin from beef posterior pituitary glands has been revealed to be chemically different from that derived from swine posterior lobes. The hormone from beef contains an arginine moiety, while that from swine contains a lysine moiety(11). The insulins from beef, swine, and sheep glands have been found to be chemically different with respect to six amino acids, although physical and biological differences are slight(12). It is not unreasonable to anticipate that similar differences may be found in corticotropin and other anterior lobe hormones of diverse animal origins. It is possible that differences in biological activity may accompany chemical diversities. A preparation recently reported by Hess and Carpenter(13) had A.A.F. activity comparable to that of corticotropin-B. Since both the animal source material and method of preparation are different in the two preparations, it is not unlikely that they may show a different spectrum of biological activities.

A second controversial issue is concerned with the question of whether adrenal ascorbic acid depletion and chromatophore expansion are due to the same or to different chemical entities. Since corticotropin-B also shows potent chromatophorotropic activity, the same reservations must be made about species differences and about the possibility of the existence of substances other than corticotropin-B which may possess predominantly either one or the other type of activity. It is entirely possible that when other substances are isolated from ACTH-concentrates, they may show spectra of biological activity different from that of corticotropin-B.

Summary. A product isolated from a pepsin digest of swine corticotropin and behaving in countercurrent distribution studies like a pure substance has been designated corticotropin-B. This product not only has very high adrenal ascorbic acid reducing activity, but is also extremely active in restoring adrenal weight in hypophysectomized rats, and has a high degree of chromatophorotropic activity.

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Some Effects of Certain Resorcinol Derivatives on Eastern Equine Encephalomyelitis Virus. Factors Influencing *in vitro* Screening Technic.* (20119)

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The present report is concerned with compounds which have a marked *in vitro* inactivating effect on the Eastern Equine Encephalomyelitis (EEE) virus and which are for the most part non-toxic, ranging in acute toxicity from 15 mg to 50 mg/100 g body weight.

The study has been divided into two phases. In the first, the conditions of interaction were investigated by noting the effects of temperature, concentration, pH and protein in the chemical-virus system. On the basis of this information, the second phase, *in vitro* screening of various compounds, was possible under simple standard conditions.

Materials and methods. The EEE virus used in this study was obtained through the courtesy of Dr. Karl Habel, U. S. Public Health Service. It was maintained at −30°C as pools of 20% mouse brain suspensions (by weight) in distilled water. Throughout the investigation, CFW mice (18-20 g) were used both as source animals and as indicators for the presence of virus. The specific nature of the virus pools was frequently checked by neutralization test with known antiserums. In preparing virus for each inactivation experiment a vial of a given pool was thawed, the emulsion diluted 4 times with sterile distilled water and centrifuged at 13,000 r.p.m. for 5 minutes in a Servall angle centrifuge, Model SS1. The supernate was diluted 5 times to a final virus dilution of 1:100 and was maintained in cracked ice until mixed with each compound to be tested. To make certain that deterioration of virus would not be confused with inactivation, the control solution was always the last one to be mixed and therefore the last to be injected into mice. Under the usual experimental conditions, the titer of the virus pool was approximately $10^{-8.0}$. Of the *14 resorcinol derivatives,* all but the 4-chloro- and 4-bromo-resorcinol† were synthesized in this laboratory. Agreement of melting points (where possible) with previous reports was taken as a criterion of purity. In the case of the substituted fluorescein dyes, purity of the compounds was established by quantitative analysis performed by an independent laboratory and by paper chromatography. The 2,7 diiodotetraiodofluorescein is a new compound and the method of synthesis of this compound as well as new methods of synthesis for the 5-nitro-beta-resorcylic acid, 3,5 diiodo-beta resorcylic acid and octoiodofluorescein were developed in this laboratory and are being reported by one of us(1).

Inactivation technic. One-half gram of the compound to be tested was partially dissolved at room temperature (22-24°C) in 2.0 ml of sodium hydroxide (1.0 M) plus 23 ml disodium hydrogen phosphate solution (M/15). Twenty-four to 30 hours later, when the inactivation experiment was carried out, this 2% solution was diluted to 1% with distilled water and the desired pH adjustment was made by the addition of hydrochloric acid or sodium hydroxide solution. To test inactivation, 1:100 dilutions of both virus and compound were mixed and the 1:200 compound-virus solution was allowed to incubate under specific experimental conditions. The incubation took place in the absence of light so that photodynamic action would not be a factor. When virus activity was to be tested at predetermined time intervals, 1.0 ml of the compound-virus mixture was diluted with 4.0 ml of 10% inactivated rabbit serum and 0.03 ml of 10-fold serial dilution was injected intracerebrally into 5 CFW mice per dilution. The LD_{50} of each chemical-virus system was thus deter-

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† These compounds were obtained from the Eastman Kodak Co.

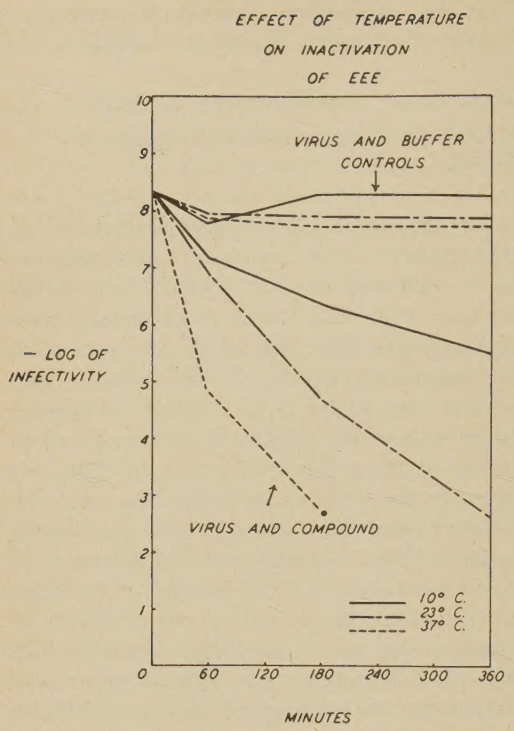

FIG. 1. Lower 3 lines represent virus and 2,7-diiodotetraiodofluorescein mixtures. Corresponding controls are represented by the upper 3 lines. Test periods are 60, 180 and 360 min. (At 37°C the testing limit was reached at 180 min.)

mined. The controls consisted of virus in buffer solution (Na_2HPO_4, M/15) adjusted to the same pH as the compound to be tested. All virus mixtures were handled in the same manner, each injection of animals per dilution taking place within 10 minutes of withdrawal of the aliquot to be tested. The inactivation effect of a given chemical was then calculated by comparing LD_{50} values of the chemical and virus with control and virus. With this technic, *in vitro* inactivation of approximately 6 logs or 1 million mouse intracerebral units could be measured.

Experimental. I. Conditions affecting inactivation of EEE virus with 2,7-diiodotetraiodofluorescein. Effect of temperature on inactivation. Using the routine technic, the course of inactivation of EEE by 2,7-diiodotetraiodofluorescein was followed at 3 different temperatures: 10°, 23°, and 37°C. The results seen in Fig. 1 show the greatest inactivation occurred at 37°C. It is noteworthy that the order of inactivation after one hour at 37°C has, for all practical purposes, proceeded to completion, since the initial LD_{50} of the chemical-virus mixture has been reduced within that time from 500 million infectious mouse units per 0.03 ml to 95 thousand infectious mouse units. This represents a reduction of infectivity of over 99%.

Effect of dye concentration. Where concentration of dye was reduced from 1.0% to 0.4% (Table I), other factors remaining constant, it was apparent that the greatest degree of inactivation occurred with the 1% solution, the highest concentration tested. The different inactivating effects of the 3 dye concentrations were more clearly seen at the 360-min. test interval.

Effect of pH. 2,7-diiodotetraiodofluorescein solution was routinely prepared so that a final pH range of approximately 6.0, 7.0, 8.0, and 9.0 was obtained in 4 aliquots. Comparable virus controls were prepared for each pH. The compound-virus and control-virus mixtures were incubated at 22°-24°C for one, 3, and 6 hours, and virus activity was measured at each of these time intervals. As can be seen from Fig. 2, the most rapid and significant inactivation occurred at pH 7.0 and 8.0. At pH 9.0 and pH 6.0, the action of dye on virus was masked because of the rapid deterioration of the control in one hour consistent with the results of other investigators(2,3).

Effect of added protein. To study the effect of protein on inactivation, 1% 2,7-diiodotetraiodofluorescein solution was adjusted to pH 8.0, as above, and diluted at room temperature (22°-24°C) with equal parts of virus in water, virus in 25% rabbit serum, or virus in 50% rabbit serum. Table II illustrates

TABLE I. Effect of 2,7-diiodotetraiodofluorescein Concentration on Inactivation of EEE Virus.

Inactivation mixture (% dye and virus)	Change of LD_{50}* with time (min.) 60	180	360
1.0	6.1	4.1	3.3
0.7	6.3	5.5†	4.7
0.4	6.5†	5.5†	5.3
Control (Na_2HPO_4, M/15) and virus	7.7	7.5	7.3

* Negative log.
† Estimated LD_{50} (no end point).

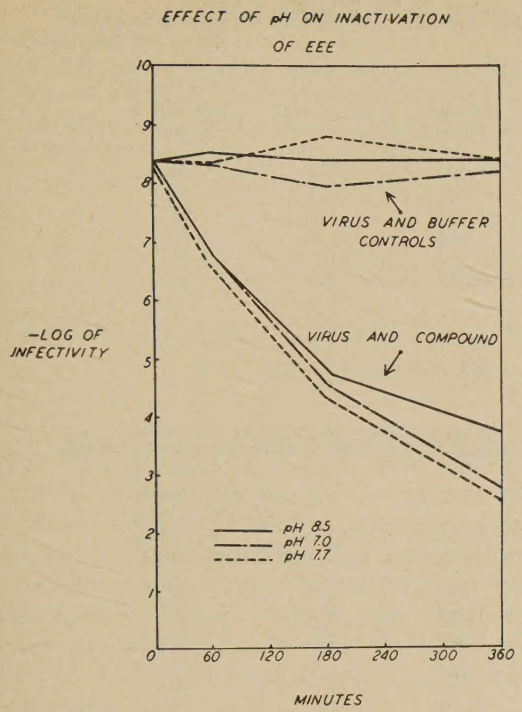

FIG. 2. Lower 3 lines represent virus and 2,7-diiodotetraiodofluorescein mixtures. Corresponding controls are represented by the upper 3 lines. Test periods are 60, 180 and 360 min.

the inverse relationship between the degree of inactivation and the amount of protein present in the dye-virus mixture. It is to be noted that the routine dye-virus mixture in water inactivated the virus at the same rate as in the previous experiment carried out under similar conditions, *i.e.*, a reduction of one and 4 logs of virus activity occurred at one and 3 hours respectively, while complete measurable inactivation was seen at 6 hours. In view of the known combining power of serum for halogenated fluoresceins(4-6), these results were not surprising.

TABLE II. Influence of Protein on Inactivation of EEE Virus by a Fluorescein Dye.

Inactivation mixture	Change of LD_{50}* with time (min.) 60	180	360
Dye and virus in:			
water	6.9	4.3	<2.5†
12.5% rabbit serum	7.9	6.9	6.3
25% " "	7.5	7.1	>6.5†
Control (Na_2HPO_4, M/15) and virus	7.9	8.3	7.9

* Negative log.
† Estimated LD_{50} (no end point).

II. Screening of resorcinol derivatives by inactivation of EEE virus. As a result of the first phase, it was apparent that a major degree of *in vitro* inactivation occurred when the EEE virus and 2,7-diiodotetraiodofluorescein were mixed at pH 8.0 and remained in contact for 3 hours at room temperature (22°-24°C). Therefore, to obtain comparative data, screening of other compounds was carried out under the same experimental conditions.

Inactivation of EEE by resorcinol derivatives. Compounds with heterogeneous groupings were selected so that information could be obtained concerning the specific effect substitution might have on the process of virus inactivation. As can be seen from Table III, of the 6 compounds tested in this group, the greatest degree of inactivation was obtained in the 3,5-diiodo-beta-resorcylic acid-virus system with an LD_{50} change from $10^{-7.9}$ to $10^{-4.7}$ within the 3-hour test period. The only other inactivation of possible significance occurred with the use of 4,6-diiodoresorcinol. As in phenolic chemistry(7), where the order of reactivity of the halogens increases as one moves down the periodic table, the bromo and chloro compounds were not active against the EEE virus under the given experimental conditions. Using the same screening technic, a group of fluorescein dyes was also tested. In Table IV, it can be seen that the iodine molecule again is a significant factor in contributing to the inactivating capacity of the fluorescein nucleus. No attempt is made to differentiate quantitatively between chlorine and bromine in this respect, because the study has been carried out on a per cent concentration rather than on the basis of mole weight. However, both bromine and chlorine were not conspicuous in their activity. It is also of interest that the largest molecule, *i.e.*, the octoiodofluorescein with a molecular weight of more than 1300, gave the most dramatic inactivation, even though its concentration was only 0.007 M.

Discussion. The present study has been limited to a simplified *in vitro* method and has attempted to demonstrate the usefulness of this technic. The need for studying various

TABLE III. Inactivation of EEE Virus by Resorcinol Compounds.*

Compound	Mole wt	Moles/ 1% sol.	LD_{50} (neg. log value)	Acute toxicity (min. lethal dose), mg/100 g body wt
5-Nitro-beta-resorcylic acid	198.12	.051	7.5	35
4-Chlororesorcinol	144.57	.069	7.3	15
4-Bromoresorcinol	189.03	.053	7.1	45
2,4-Dihydroxyacetophenone	152.14	.066	7.5	22
3,5-Diiodo-beta-resorcylic acid	405.96	.025	4.7	>50
4,6-Diiodoresorcinol	361.95	.028	6.5	40
Control (Na_2HPO_4, M/15)			7.9	

* pH of compounds = 8.0.
Contact period between virus and compound = 3 hr. Exp. at 22°-24°C.

TABLE IV. Inactivation of EEE Virus by Fluorescein Dyes.*

Compound	Mole wt	Moles/ 1% sol.	LD_{50} (neg. log value)	Acute toxicity (min. lethal dose), mg/100 g body wt
Fluorescein	332.30	.030	8.3	50
4,5-Diiodofluorescein	584.14	.017	6.3	40
Tetrachlorofluorescein	470.14	.021	5.9	35
Tetrabromofluorescein	647.98	.015	7.5	40
Tetraiodofluorescein	836.28	.012	5.7	45
4,5-Diiodotetraiodofluorescein	1088.12	.009	5.1	35
2,7-Diiodotetraiodofluorescein	1088.12	.009	6.3	30
2,4,5,7-Octoiodofluorescein	1339.96	.007	3.5	40
Control (Na_2HPO_4, M/15)			8.3	

* pH of compounds = 8.0.
Contact period between virus and compound = 3 hr. Exp. at 22°-24°C.

conditions of virus-chemical interaction is apparent since failure to consider this factor has resulted in negative reports for such acid dyes as eosin, eosin B, eosin Y, erythrosin and rose bengal(8-11).

From the structure of the molecules observed in this study, it seems that the most active molecules contain iodine atoms as shown by the high inactivating capacity of 4,6-diiodoresorcinol, 3,5-diiodo-beta-resorcylic acid and octoiodofluorescein. That the inactivation by the iodinated resorcinols is not due to free iodine is evident from the following. Greater inactivation occurred at pH 7.0 and 8.0 than at 9.0, the pH at which iodine is more easily freed. Furthermore, resorcinol derivatives without iodine have been found to possess great inactivating capacities(12). However, iodination enhanced the inactivating capacity of resorcinol derivatives to a greater extent than other forms of halogenation.

Conclusions. 1. Various resorcinol derivatives of a non-toxic nature have been shown to be active against EEE virus under *in vitro* conditions. 2. Some factors affecting inactivation of EEE virus by 2,7-diiodotetraiodofluorescein have been presented and their importance in an *in vitro* study on compound-virus interaction stressed. 3. An *in vitro* screening technic has been demonstrated permitting the study of the effect of compound configuration on virus activity.

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Chelators and Resinous Exchangers. (20120)

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Cation exchange resins of the carboxylic and sulfonic types have found wide application in medical practice for the purpose of sodium removal in cardiac decompensation cases. A major deterrent to a still broader use is the bulk of such agents required for therapeutic efficacy. Clearly, any improvement in capacity would tend to reduce required intake and hence increase patient cooperation.

It was postulated that capacity would be augmented through the removal by chelating agents of polyvalent cations leaving only the monovalent cations for exchange with the resin. Even if this ideal state could be approximated, improvement should ensue. Ethylenediamine tetra-acetic acid or any similar agent will chelate only with polyvalent cations and it is these ions which possess the greater affinity for cation exchange resins, and therefore reduce *in vivo* capacity.

Experimental. The experimental design paralleled in large measure that employed by McChesney and his associates(1-3), Ch'en and Freeman(4), and others. Natrinil, a commercial product, which is a purified cation exchanger carboxylic in a very finely divided state (90% through 200 mesh), 20% in the potassium cycle, and 80% in the hydrogen cycle was used. The *in vitro* capacity was *ca.* 10.0 meq./g. The ethylenediamine tetra-acetic acid was a very pure sample which was used without further purification. The diet had the following composition:

	g
Casein	1500
Salt mix (Hubbel, Mendel, Wakeman)	300
Crisco	1050
Sucrose	3000
Cod liver oil	225
Sodium chloride	75
Yeast	600
Roughage or resin as desired	750
Total	7500

As indicated above, any quantity of roughage may be replaced by resin as the experiment would require. In this way the total quantity of bulk was kept roughly constant. The resin was incorporated into the diet at a 5% level and the EDTA when used at a 1% level.

A pair of rats was placed in each metabolism cage and given the experimental diet and water *ad libitum* for 3 days, were then placed in clean cages and the diet was continued with careful check on the food consumption. The feces were collected every day and pooled until the end of the experiment. The urine was allowed to accumulate in containers containing 3 ml of concentrated nitric acid. The rats were kept on this diet for 4 days and were then placed in clean cages on the same or new diet for the conditioning period (3 days). The collection was made in the usual way. The feces were thoroughly dried at 80-105°C, then ground and thoroughly mixed.

After a review of the literature on flame photometry it was decided to check the results reported by Mosher *et al.*(7) and the agreement was good. Methods were then developed using the idea of radiation buffers as proposed by West *et al.*(8). It was found that the results obtained by this procedure were completely reproducible. Methods for the various elements are summarized briefly below. The urine was checked to make certain that it was

TABLE I. Effect of Ethylenediamine Tetra-Acetic Acid on *In Vivo* Capacity of Cation Exchange Resin. (All values mg/100 g of ingested food.)

	EDTA	Carboxylic cation exchange resin	Combined resin + EDTA	Controls
Fecal Na	86 (4)	127 (5)	346 (2)	75 (20)
	—	123 (10)	176 (10)	51 (19)
	—	175 (7)	245 (7)	67 (20)
	—	164 (7)	211 (7)	—
Urinary Na	536 (4)	388 (5)	476 (2)	458 (20)
	—	312 (10)	202 (10)	350 (19)
	—	249 (7)	189 (7)	309 (20)
	—	244 (7)	194 (7)	—
Fecal K.	110 (4)	138 (5)	409 (2)	114 (20)
	—	124 (10)	234 (10)	84 (19)
	—	139 (7)	273 (7)	81 (20)
	—	127 (7)	221 (7)	—
Urinary K.	593 (4)	674 (5)	823 (2)	595 (20)
	—	571 (10)	405 (10)	436 (19)
	—	478 (7)	398 (7)	438 (20)
	—	503 (7)	460 (7)	—
Fecal Ca	487 (4)	639 (5)	899 (2)	741 (20)
	—	729 (10)	529 (10)	641 (19)
	—	584 (7)	541 (7)	705 (20)
	—	638 (7)	462 (7)	—
Urinary Ca	44 (4)	21 (5)	38 (2)	14 (20)
	—	16 (10)	18 (10)	27 (19)
	—	14 (7)	15 (7)	29 (20)
	—	17 (7)	22 (7)	—
Fecal Cl.	108 (4)	102 (5)	194 (2)	118 (20)
	—	57.8(10)	179 (10)	61 (19)
	—	113 (7)	189 (7)	104 (20)
	—	92 (7)	152 (7)	—
Urinary Cl.	954 (4)	716 (5)	903 (2)	815 (20)
	—	626 (10)	552 (10)	630 (19)
	—	463 (7)	490 (7)	532 (20)
	—	626 (7)	604 (7)	—

Figures in parentheses represent No. of pairs of rats in each exp. leading to avg value stated.

definitely acid and was then diluted to 200 cc (urine stock solution). The "sodium buffer" was prepared by dissolving 0.5 mole each of potassium, calcium and magnesium chlorides in one liter of solution. In a similar fashion the "potassium buffer" was 0.5 molar in sodium, calcium and magnesium chlorides, while the "calcium buffer" was 0.5 molar in sodium, potassium and magnesium chlorides. For the determination of sodium in urine, a standard curve was prepared using 1 ml of sodium buffer per 100 ml and known amounts of sodium chloride. The unknown was then prepared in the same way and its value determined from the standard curve. The method for potassium was similar to sodium except that the potassium buffer was used at the level of 4 ml of buffer per 100 ml. For the analysis of calcium in urine a new method, the validity of which will be established elsewhere, is described. Five ml of urine were pipetted into a 25 cc graduated cylinder; 0.5 ml of calcium buffer was added and then 3.0 ml of 1.0 N ammonium hydroxide. This mixture was diluted to 25 ml, allowed to stand for 10-15 minutes and then filtered. The clear filtrate was read on the flame photometer. The method for chloride in urine was that of Sendroy modified by Van Slyke and Hiller(9). Two g of dry, ground feces were placed in a Vycor crucible, moistened with 1 cc of 6 N sulfuric acid, evaporated to dryness, charred, ashed in a muffle furnace at 500 to 550°C, cooled. One cc of conc. HCl added and the mixture heated. One cc of conc. HNO_3 was added and this was heated. Five ml of distilled water were added and the mixture heated to boiling. It was then diluted to 25

ml and filtered. The filtrate was used for analysis of sodium, potassium and calcium in a manner similar to that described for the urine. The method for chloride in feces was essentially the open Carius method with a modified Volhard-Harvey titration as described by Peters and Van Slyke(10). Phosphorus in the urine and feces was determined as outlined in Hawk, Oser, and Summerson(9).

Results and discussion. From Table I, it is seen that ethylenediamine tetra-acetic acid given alone does not modify sodium, potassium or chloride content but does reduce calcium content of feces. The chelator tends to increase urinary calcium and chloride with no modification in sodium or potassium. In explanation, it is proposed that calcium as a result of its chelation is removed from insoluble salts normally present in the intestine and rendered soluble with resultant absorption and subsequent excretion via the kidney. The facilitation of chloride absorption caused by the EDTA, as reflected in increased urinary excretion, results from reduction by EDTA of calcium cation concentration normally restricting some chloride ions to the intestinal content.

The results reported with the cation exchange resin alone are in large measure similar to those reported by other investigators(1-4) and need no comment. The combination of EDTA with the cation exchange resin compared to resin alone resulted in a marked increase in the capacity of the resin for sodium and potassium removal. Calcium excretion in the feces was decreased by the concomitant administration of EDTA with the resin. The proposed explanation of these findings is that calcium is chelated as when EDTA is given alone and as a result absorbed or rendered non-reactive. This frees the full capacity of the resin for exchange with monovalent cation, and the result is increased capacity. The degree of this effect will be associated with the relative abundance of polyvalent and monovalent cations in the intestinal content, the higher the relative polyvalent ion concentration the more marked will be the potentiation manifested by the EDTA.

EDTA given alone does not modify fecal chloride but does increase urinary chloride. Given with a resin, it modifies the resin effect in that it reduces urinary chloride and increases fecal chloride. It will be recalled that anion exchange resins modify the altered anion excretion caused by cation exchangers in that urinary chloride excretion is decreased and fecal chloride excretion is increased(5,6). Whether or not EDTA reduces the hyperchloremia resulting from cation exchange resin administration remains to be found. It may well be that the combination will reduce the tendency toward the development of acidosis associated with resin usage.

Summary. Ethylenediamine tetra-acetic acid given with a carboxylic cation exchange resin increases the capacity of the resin for monovalent cation removal. A concept is advanced in explanation of this effect.

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Fate of Gentisic Acid in Man as Influenced by Alkalinization and Acidification. (20121)

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Gentisic acid in the form of its sodium salt has been recently advocated as an anti-rheumatic and analgesic in place of salicylates. It was first pointed out by Neuberg(1) that gentisic acid could be recovered from the urine of subjects receiving salicylates. It was not, however, until 1948 that Ragan and Meyer(2) suggested that gentisic acid might be the active anti-rheumatic factor of salicylates in view of its marked ability to inhibit hyaluronidase. Although the anti-rheumatic activity and analgesic action of gentisic acid has been confirmed(3), there is considerable doubt that gentisic acid *per se* is the sole active component of salicylate metabolism. Since gentisic acid possesses these properties in its own right, it was thought advisable to investigate its fate after oral administration in man in order to determine whether gentisic acid behaves in the body like salicylates. This report will consider primarily the urinary excretion of gentisic acid as compared with salicylates when influenced by acidification and alkalinization.

Method. Groups of subjects without renal or gastrointestinal disease were given 300 mg of sodium gentisate or sodium salicylate orally 3 times daily for 7 days to assure saturation before samples were collected for analysis. The drug was then continued and 24-hour urines were collected for 3 consecutive days. Similar studies were repeated but with the administration of sodium gentisate or sodium salicylate in combination with sodium bicarbonate (0.6 g), dilute hydrochloric acid (1 cc of 0.1 N, U.S.P.), or ammonium chloride (enteric coated 1 g) 3 times daily. The salicylates were then determined by the method of Lester, Lolli, and Greenberg(4). An aliquot of the 24-hour urine was acidified with hydrochloric acid and then hydrolyzed. The hydrolysate was then extracted with ethylene dichloride which was in turn extracted with water. Ferric nitrate was added to the aqueous extract to produce a blue color. The method of determining gentisic acid was a modification of the methods advocated by Lutwak-Mann(5) and Kapp and Coburn(6). The urine was acidified with hydrochloric acid and extracted with ether until the urine failed to give the alkali test (*i.e.,* urine plus sodium hydroxide would give a pink color if any gentisic acid were present). The ether extract was washed with sodium bicarbonate until colorless. The aqueous layer was reextracted with acid and ether. If salicylate was known to be present, chloroform and benzene were used to remove the salicylate. The ether layers were dried, taken up in potassium acid phthalate, and ferric chloride added to produce a blue purple color which could then be measured colorimetrically.

Results. The data on gentisic acid recovery, whether combined or free and the influence of alkalinization or acidification are summarized in Table I. The results of similar studies with salicylates, representing only the total salicylate and its gentisic acid metabolite are indicated in Table II.

We were able to recover approximately 62% of the gentisic acid when the sodium gentisate was administered alone. Of this, approximately 42% existed in the free form. The simultaneous administration of sodium bicarbonate increased the total excretion of gentisic acid by about 35% so that we were able to recover 84% of the gentisic acid administered in the form of its sodium salt. It is of interest to note that although the amount of free gentisic acid recovered was increased by alkalinization, there was no change in the relative proportion of free to the total amount eliminated (Table I).

With acidification produced by administering dilute hydrochloric acid, there was a decrease in the total excretion of gentisic acid at the expense of the combined, the free remaining unaltered. However, when acidifica-

TABLE I. Influence of Alkalinization and Acidification on Recovery of Gentisic Acid. Data present ranges of excretion in patients for 3 consecutive days of urine collection. Data for 3 days averaged. Values represent mean of average excretion for each individual group as indicated.

Group	Patients No.	Total G.A.* recovered, mg		Free G.A.* recovered, mg		% G.A.* recovered		Free/total G.A.* × 100	% change due to alkalinization and acidification	
		Range	Mean	Range	Mean	Total	Free		Free	Total
G.A.*	9	500–551	533	210–241	225	62.4	26.3	42.0	—	—
G.A. + sodium bicarbonate	6	714–726	721	331–346	336	84.4	39.2	46.3	+49.0	+35.2
G.A. + HCl	6	423–471	442	224–246	235	51.7	27.5	53.1	+ 4.5	—17.1
G.A. + NH_4Cl	6	488–509	501	252–257	255	58.7	29.8	50.7	+13.3	— 5.9

* G.A. = Gentisic acid.

TABLE II. Influence of Alkalinization and Acidification on Recovery of Salicylate and Its Gentisic Acid Metabolite. 6 patients in each series. Data present ranges of excretion in individual patients for 3 consecutive days of urine collection. Data for 3 days averaged. Values represent mean of average excretion for each individual group as indicated.

Group	Total S* recovered, mg		% recovered of ingested S*	Total G.A.† recovered, mg		Free G.A.† recovered, mg		% G.A.† recovered of S* ingested		Free/total G.A.† × 100	% change G.A.† recovered due to alkalinization and acidification		% change S* recovered due to alkalinization and acidification
	Range	Mean		Range	Mean	Range	Mean	Total	Free		Free	Total	
S*	594–633	617	68.6	52–58	54.6	4.6–5.1	4.8	6.1	.53	8.8	—	—	—
S + $NaHCO_3$	787–794	790	87.6	75–81	78.2	6.7–7.8	7.2	8.4	.79	9.2	+49.1	+37.7	+27.8
S + HCl	385–496	457	50.7	46–49	48	4.6–5.2	5.0	5.3	.55	10.4	+ 3.7	—13.1	—26.1
S + NH_4Cl	418–440	431	47.8	44–47	46	3.9–4.4	4.1	5.1	.46	9.0	—13.2	—16.4	— 30.3

* S = Salicylate.
† G.A. = Gentisic acid.

TABLE III. Statistical Data.

Group*	Total gentisic acid — Mean, mg	Range, mg	SD,† mg	"T"‡
1. G.A.	531	280–805	±124.9	—
G.A. + $NaHCO_3$	721	646–802	±153.9	7.37
G.A. + HCl	431	224–552	± 73.8	3.82
G.A. + NH_4Cl	493	443–631	± 60.2	1.05
2. S	622	538–685	± 48.73	—
S + $NaHCO_3$	787	709–858	± 71.5	11.6
S + HCl	478	344–624	± 74.1	6.3
S + NH_4Cl	435	380–509	± 44.2	13.6
3. G.A. recovered:				
from S	48.8	40.5–59.8	± 5.62	—
from S + $NaHCO_3$	70.9	59.3–86.8	± 7.23	9.1
from S + HCl	43.2	35.0–50.1	± 4.09	3.09
from S + NH_4Cl	41.5	33.7–47.2	± 3.71	4.06

* G.A. = Gentisic acid; S = Salicylate.
† SD = Stand. dev.
‡ "T" = Statistical symbol to indicate significance.

tion was obtained by the use of ammonium chloride, a lesser effect upon combined gentisic acid was demonstrated than with hydrochloric acid.

Approximately 68% of the administered sodium salicylate was recovered in 24 hours from the urine. Approximately 6% of the administered sodium salicylate was recovered as gentisic acid. Upon alkalinization there was a 28% increase in the total salicylate excreted so that we were able to recover approximately 88% of the drug administered (Table II). Alkalinization also affected the excretion of the degradation product, gentisic acid, since its excretion increased to 8.4% which represents an increase of approximately 38%. Although there was an absolute increase in the free gentisic acid, there was no alteration in the proportion of free to combined obtained prior to alkalinization.

Acidification with dilute hydrochloric acid resulted in a decrease of total salicylate so that only 51% of the administered amount was recovered. This decreased excretion was also reflected in the total gentisic acid recovered, but again at the expense of the combined.

In contrast to the effects of ammonium chloride on gentisic acid which were insignificant, the simultaneous administration of ammonium chloride with sodium salicylate resulted in typical changes associated with acidification.

Table III presents a statistical evaluation of the data. The results with gentisic acid are statistically significant for alkalinization with sodium bicarbonate and acidification with hydrochloric acid. The data obtained for salicylate elimination and the elimination of its gentisic acid metabolite are also significant.

Discussion. In the main, gentisic acid followed the same pattern of excretion as salicylate. However, when the gentisic acid produced by salicylate administration was compared to the administration of sodium gentisate alone, there was noted a significant difference in the proportion of free to total gentisic acid recovered. Where the amount of free gentisic acid obtained from salicylate administration was approximately 10% of the total gentisic acid recovered; with the administration of the gentisate alone, free gentisic acid comprised approximately 42% of the total. This proportion of free to total obtained from the salicylate administration remained unaltered under the influence of alkalinization and acidification. We have no explanation as to why the proportion of free gentisic acid to combined is much lower when obtained from salicylate administration than from sodium gentisate alone.

If the anti-rheumatic and analgesic effects of gentisic acid are dependent upon the amount of free gentisic acid present in the body, it is apparent that the amount of the free gentisic acid formed from salicylate would not be of sufficient amount to be of clinical significance. Too small amounts of free gentisic acid are present after salicylate ad-

ministration to account for the latter's pharmacologic effects. The amount of free gentisic acid present in the body cannot be changed by alkalinization or acidification and therefore the effects noted are of pharmacologic but not clinical interest.

Conclusions. 1. The recovery of gentisic acid and salicylate were similar, approximating 62 and 68% respectively of the administered drugs, and 84 and 88% respectively, following alkalinization. 2. Alkalinization increased the combined and free forms of gentisic acid eliminated but did not alter their relative proportions. 3. The amount of gentisic acid recovered from salicylate administration was increased with alkalinization without alteration in the proportion of free to combined gentisic acid obtained with salicylate alone. 4. Whereas the free gentisic acid represented approximately 42% of the total gentisic acid recovered when administered alone, the amount of free gentisic acid obtained from salicylate administration represented approximately 10% of the total gentisic acid. 5. Acidification with dilute hydrochloric acid, but not ammonium chloride decreased the total excretion of gentisic acid at the expense of the combined, the free remaining unaltered. 6. Acidification with both dilute hydrochloric acid and ammonium chloride decreased the total excretion of salicylate and the combined gentisic acid metabolite, the proportion of free to combined gentisic acid remaining unaltered.

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Histochemical Studies of Thermal Injury on Rat Skin.* (20122)

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During the course of an histochemical study of thermally injured rat skin, a unique differential staining property of heat-altered collagen was observed.

Methods. The abdomen of young adult male rats was exposed to various temperatures between 50°C and 80°C for 1-2 minutes in a thermostatically controlled water bath. The area exposed was limited by a shielding device. Immediately following the thermal exposure, skin blocks were excised which included both the exposed and shielded areas. These were immediately fixed in Carnoy's fixative to prevent solution of water soluble substances present as normal constituents of skin or as a result of the heat treatment.

Results. Paraffin sections (4 μ) were stained for 24 hours at room temperature by an aqueous solution of aniline blue (C.I. No. 707) and a mordant, phosphomolybdic acid. Both the normal and the heat-altered collagen of the rat skin stained an intense blue. When the hyperthermic episode ranged between 50 and 60°C, the heat-altered collagen was indistinguishable from that of normal unheated skin. At higher temperatures (70°C and 80°C) the heat-altered collagen showed a variable loss in structure, due to homogenization and swelling of the individual bundles. Orange G (C.I. No. 127) added to the above

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TABLE I. Staining of Rat Skin Collagen. Effect of solvents and dye combinations.

Exposure, °C	Stain soln.* Dyes	Solvent	Treatment	Collagen stain Normal	Burn
50	1, 2, 3	M	0	B†	B
55	1, 2, 3	M	0	B	B
60	1, 2, 3	M	0	B	B
70	1, 2, 3	M	0	B	Y
80	1, 2, 3	M	0	B	Y
50–80	1, 2, 3	W	0	B	B
70	1, 3	M	0	0	Y
80	1, 3	M	0	0	Y
70	2, 3	M	0	B	0
80	2, 3	M	0	B	0
50–80	1, 2, 3	M	Saline phosphate	B	B
50–80	1, 2, 3	M	Trypsin	B	B (a)

* 1 = Orange G; 2 = Aniline blue; 3 = Phosphomolybdic acid; M = Methanol; W = Distilled water.

† B = Blue; Y = Yellow; (a) = Any collagen remaining, following tryptic digestion.

aqueous staining solution serves as a cytoplasmic and keratin stain.

When, however, the above stains (aniline blue, phosphomolybdic acid) were dissolved in absolute methanol and duplicate tissue sections stained for 24 hours, there was a striking difference in the staining properties of normal collagen and those of collagen which has been brought to temperatures of 70°C or above. In these circumstances, the thermally-altered collagen stained intensely with Orange G in contrast to the normal unheated collagen, which is still stained dark blue by the aniline blue. At the periphery of the heat-altered area, many of the collagen bundles appeared structurally unchanged to the extent that they were not swollen and had not lost their fibrillar appearance. In this region, where the temperature rise was not as great, collagen stained blue-green. On inspection at high magnification, this was seen to be due to partial uptake of both Orange G and aniline blue by individual collagen bundles. Sections stained with either dye plus phosphomolybdic acid in absolute methanol showed the dye uptake of normal and heat-altered collagen to be mutually exclusive, *i.e.*, normal collagen was unstained by Orange G and heat-altered collagen was unstained by aniline blue. Where tissues were prepared in aqueous fixatives and stained by the methanol staining method, differential staining of normal and heat-altered collagen did not occur. These sections stained blue throughout.

From these studies it was inferred that the hyperthermic episode had rendered some component of the dermal collagen water soluble. In addition, this component was altered in its staining reaction to aniline blue and failed to be stained by it. Rather, it now was stained by Orange G. When microscopic sections were extracted with distilled water, M/15 Sörenson Buffer plus M/50 NaCl at pH 7.7, or M/6 NaCl alone, the normal and heat-altered collagen both stained blue in the methanol dye solution given above. The heated areas, however, showed some attenuation and loss of stainable collagen. These observations further substantiated the presence of an aqueous soluble collagen derivative, the result of the thermal exposure. That the previously differentially stained heat-altered collagen bundles became aniline blue positive following various aqueous extractions suggested a surface alteration of the collagen bundles consistent with partial denaturation. As such, this reaction might be interpreted as a partition of the effect of heat on collagen.

A further partition was observed by the staining of sections which were treated with trypsin (1 mg/ml in M-15 Sörenson Buffer-M/50 NaCl at pH 7.7) for one hour at 37°C. Sections of skin previously exposed at temperatures from 60°C to 80°C showed degradation and attenuation of collagen bundles which were most marked at the highest temperature (80°C). In some cases, normal collagen structures were completely obliterated by the tryptic action. On the other hand, the staining reaction of normal unheated portions of

TABLE II. Differential Staining of Heat-Altered* Collagen by Azo Dyes.

Dye	C.I. No.	No. of chemical groups Azo	SO_3^-	Differential stain
Orange I	150	1	1	+
II	151	1	1	+
III	142	1	1	+
IV	143	1	1	+
G	127	1	2	+
Amaranth	184	1	3	+
Sudan III	248	2	0	—
Biebrich scarlet	280	2	2	+
Chlorazol black E	581	3	2	—

* Heat exposure 70°C or more. Specimens excised 90 sec. after heating.

collagen on the sections was deep blue and similar to that seen in the untreated controls. In both portions, all remaining collagen stained blue. These observations served to verify the specificity of the Orange G for staining heat-altered areas of collagen.

In addition, the effect of trypsin further partitions the heat-induced alterations of collagen. In sections failing to stain differentially with Orange G (*e.g.*, 60°C), there was, nevertheless, a loss of collagen substance by enzyme treatment. This effect perhaps demonstrates collagen that has been sufficiently altered by the thermal exposure to render it hydrolyzable by trypsin but still quantitatively not degraded enough to yield the Orange G aqueous soluble products formed at higher temperatures or times of exposure.

Preliminary studies of the specificity of the azo dye uptake in methanol solutions are presented in Table II. These results showed that mono-azo and di-azo dyes with mono, di, and, trisulphonated end-groups all stain heat-altered collagen differentially. The triazo, disulphonated dye, chlorazol black E, failed to stain the heat-altered collagen. In this situation, the heated area was completely unstained. Non-sulphonated azo dyes did not stain collagen at all, while the trisulphonated diazo dye, amaranth, appeared to differentially stain heat-altered collagen, even more intensely than Orange G (di-azo, disulphonated).

These preliminary studies are presented at this time because of their possible usefulness in the understanding of collagen degradation during thermal injury.

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Circulatory Changes in the Hamster's Cheek Pouch Associated with Alimentary Lipemia.* (20123)

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Swank(1) showed that the red blood cells in humans and dogs, when observed in whole blood smears in dark field illumination, have a tendency to aggregate, be adhesive to one another, and be distorted 4 to 9 hours after a large fat meal (2 to 4 g/kg). These changes in the suspension stability of the blood were usually first detected about an hour after the chylomicron response had passed its peak, and they increased, sometimes becoming very marked, as the the visible lipemia lessened. With complete clearing of the visible lipemia 9 to 12 hours after the fat meal the suspension stability of the blood returned to normal. The altered suspension stability of the red blood cells was accompanied by a tendency of the chylomicrons to aggregate, by changes in the sedimentation rate and hematocrit(1), and by alterations in the pattern of paper chromatograms (Swank, Franklin and Quastel)(2).

In the present paper the effects of large fat meals on the intact circulation of the blood

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in the cheek pouch of the golden hamster will be reported.

Material and methods. Golden hamsters weighing 70 to 100 g were fed via stomach tube cream fat meals varying in size from 4 to 15 g/kg body weight. At variable intervals after the fat meal the animals were anesthetized with 20% urethane intraperitoneally and one cheek pouch was inverted and prepared for visualization by the method of Fulton, Jackson and Lutz(3). In most cases one membrane of the pouch was removed, but for control purposes a number of pouches were studied with the membranes intact to determine the effect of the increased trauma and exposure occasioned by the removal of one membrane. The pouch was immersed in Ringer's solution and maintained at a temperature of 37.5°C. Observations were made at magnification of 750 X to 1350 X but for cinephotomicrography only the 50 X water immersion objective was used. In a few early studies black and white film was used for cinephotomicrography but usually the circulatory changes were recorded in color. The movies were all taken at 32 frames/second, but they were studied at 16 frames/second to allow better visualization of the blood elements. In a number of hamsters a small polyethylene tube was placed in the femoral vein so that injections could be made and frequent blood samples could be obtained. The blood samples were observed in dark field illumination to determine the chylomicron counts. For control purposes a number of animals were studied without inserting the tube. This study is based on observations in approximately 100 hamsters.

Results. A. Circulation in the cheek pouch of normal hamsters. Our control hamsters consumed a diet of mixed grain, vegetable greens, and fox chow (approximately 20% of the total calories of fox chow are furnished by fat; the vegetable greens and mixed grain which made up a large part of the diet contain very little fat). In none of our control studies of the cheek pouch of the hamster was stickiness or aggregation of the red blood cells noted. A variable amount of adhesiveness of the white blood cells to the walls of the blood vessels, and an occasional clump of platelets were observed. This usually increased after 3 or more hours of exposure and observation of the pouch, at which time petechial hemorrhages also began to appear. Occasional groups of red blood cells in rouleaux formation were also observed in normal pouches. These usually consisted of no more than 2 to 3 red blood cells, and the individual cells were clearly distinguishable and readily separated from one another when the flow of blood changed direction or was interrupted by a more rapid flow in anastomosing vessels. Even when the flow was greatly slowed and even stopped by the production of shock, by anaphylaxis, or by the injection of the histamine liberator 48/80, no increased tendency to adhesiveness or aggregation of the red blood cells was noted.

B. Circulation in the cheek pouch of lipemic hamsters. It was found that the hamsters usually developed a greater alimentary lipemia if they had been on a high fat diet (approximately 50% of the calories were furnished by fat) for a period of some days. Most of our animals had been on such a diet for one to three weeks prior to observation and frequently this was supplemented by a daily feeding of cream for 2-4 days prior to visualization of their pouches. We have, however, observed a significant lipemia with changes to be described in the circulating red blood cells following a single fat meal without prior high fat feedings. On the *day for visualization,* the pouch was exposed 3 to 6 hours after the fat meal. No change was observed in the red blood cells during the period of increasing visible lipemia. When the chylomicron count began to fall, usually 4 to 7 hours after the fat meal, changes in the suspension stability of the red blood cells developed. First the red blood cells appeared to be slightly adhesive to one another, and here and there a red blood cell stuck momentarily to the wall of a capillary or venule or to a white blood cell which was adherent to a vessel wall. The tendency to rouleaux formation increased; the size of these formations greatly increased and the cells showed a definite tendency to remain together under circumstances which would otherwise have separated them. Simultaneously the speed of the circulation decreased. This

tendency toward aggregation gradually became much greater and in capillaries and venules red blood cells began to aggregate in irregular small clusters of four to 12 or more cells. In many vessels the flow of blood became extremely slow, and in many of these the flow soon stopped completely. These changes became first evident and remained most pronounced in the venules. When the changes just described became pronounced the aggregates of red blood cells assumed an amorphous, homogeneous appearance and the outlines of the individual cells could no longer be distinguished clearly. Frequently the red blood cells had the appearance of being covered by a "doughy" opaque material. In those vessels in which the rate of flow was greatly reduced, particularly in the venules and smaller veins, the blood flowed in great "chunks". When adherent cells separated one could sometimes see them elongated as if stretched out and on a few occasions thin strands could be made out attaching them together (Fig. 6 in paper by Swank(1)). Sometimes clumps of platelets and platelets adhering to red blood cells were observed at this stage. As a rule, however, no increased adhesiveness of the white blood cells was noted unless the pouch had been opened. Petechial hemorrhages usually appeared in those pouches in which adhesiveness of the red blood cells was observed; but they were seen later and were less numerous than in our control hamsters, sometimes being absent after as long as 8 hours of exposure. Some degree of aggregation and adhesiveness of the red blood cells and slowing of the circulation has been observed in all of the hamsters which developed a significant lipemia. These changes have never been seen in any of our control animals.

With clearing of the visible lipemia 7 to 10 hours after the fat meal the circulation became more rapid and the adhesiveness of the red blood cells became less marked. In many vessels in which the circulation had stopped for some hours, the sticky masses of cells were seen slowly oozing out of the vessels into more rapid flowing streams of blood. Occasionally one saw the circulation re-established suddenly in a capillary loop. The circulation has never returned entirely to normal during the same period of observation. This is probably due in part to trauma to the pouch resulting from its long exposure. However, prolonged exposure *per se* causes no change in the adhesiveness of red blood cells. A number of hamsters with circulatory changes as marked as just described were allowed to recover and their pouches were observed several days later. The circulation was then normal.

C. Circulation in cheek pouch after injections of gelatin, egg albumin and dextran. We have seen changes in the suspension stability of the red blood cells similar to those associated with lipemia after intravenous injections of small amounts of high-molecular-weight hydrophilic colloidal solutions of gelatin, egg albumin, and high-molecular-weight dextran. In these experiments the degree of change varied directly with the amount injected and could be reversed toward normal by subsequent injection of low-molecular-weight dextran, as shown previously by Thorsen and Hint(4).

Discussion. The changes in the suspension stability of the blood which we have observed in the cheek pouch of the hamster after large fat meals have some features in common with the changes noted by Lister(5) in the injured wing of the bat, and referred to as "sludging" by Kniseley *et al.*(6) when seen in the monkey in the terminal stage of malaria. Lutz and his coworkers(7) have not observed sludging in infectious disease, in stasis of the circulation and in the many other experimental studies which they have made of the hamster's cheek pouch. Lutz(7) appears to doubt the validity of some of Kniseley's reports because he thought that Kniseley had "extended his concept, without crucial evidence, to include blood flow characteristics which differ from those of the sludged blood he originally defined."

The significance of the adhesiveness and aggregation of the red blood cells which occurs after a large fat meal, and the mechanism of these changes are unknown. They have been discussed elsewhere(1,8). If it can be shown that a significant increase in the viscosity of the blood and hypoxia of the tissues can re-

sult from alimentary lipemia then this biological mechanism is of considerable physiological and probably of pathological significance. One can only speculate concerning the importance of this mechanism in human disease, particularly in atherosclerosis(9), in vascular thrombosis(10), and in multiple sclerosis(8).

Conclusion. Following large fat meals changes occur in the circulation of the cheek pouch of the golden hamster. These changes consist of an increased adhesiveness and aggregation of the red blood cells accompanied by a slowing and even cessation of flow of the blood in the exposed pouch. They appear after the peak of the lipemia has been passed, and develop to their maximum as the lipemia clears. After complete clearing of the lipemia the suspension stability of the blood returns toward normal, and the circulation again assumes a normal appearance.

A 20 minute 16 mm colored cinephotomicrograph with titles has been prepared showing the circulatory changes described in this paper.

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Concerning the Mechanism of Action of Enterokinase.* (20124)

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Lipotropically active fractions from pancreas have been prepared by Dragstedt *et al.* (1) and by Entenman *et al.*(2). Recently Bosshardt *et al.*(3) obtained a fraction possessing lipotropic activity from the residue remaining after extraction of insulin from bovine pancreas. György and coworkers(4, 5) investigated the properties of Bosshardt's preparation and concluded that the lipotropic activity of this fraction depended upon the *in vivo* liberation of a proteolytic enzyme. They established that the lipotropic activity of this fraction was destroyed by boiling and that the fraction itself had no proteolytic activity *in vitro,* but became proteolytic after an inhibitor had been removed. They tentatively identified the inhibitor as pancreatic trypsin inhibitor, the enzyme as trypsin, and the lipotropic fraction as a complex between the enzyme and the inhibitor together with an excess of the free inhibitor. They further showed that the action of the inhibitor could be overcome by a substance present in duodenal juice and presumed to be enterokinase. The latter finding led them to conclude that enterokinase, besides having the well known ability of transforming trypsinogen into trypsin, is capable of overcoming the action of trypsin inhibitor on trypsin.

Since crystalline trypsin, crystalline pan-

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creatic trypsin inhibitor, and crystalline trypsin-trypsin inhibitor complex as well as purified enterokinase were available in this laboratory, it was decided to check whether the postulated new action of enterokinase could be detected in relatively pure systems.

Experimental. Crystalline trypsin, crystalline pancreatic trypsin inhibitor, and crystalline trypsin-trypsin inhibitor complex were prepared according to the methods of Kunitz and Northrop(6). Each of these substances was recrystallized at least 3 times. Stock solutions of appropriate strength were made using the previously established factors for the specific absorption at 280 mμ(7). Purified enterokinase was prepared according to Kunitz (8). We also had a sample of enterokinase prepared by Dr. Kunitz in 1940 and kindly supplied to us on a previous occasion. This sample still showed about 1/7 of its original activity, corresponding to 6 E.K.U. (Kunitz) per mg. Crystalline trypsinogen which was freed from the trypsin inhibitor by purification with trichloroacetic acid was used as substrate in the determination of enterokinase activity (9). The proteolytic activity was determined by the spectrophotometric method of Kunitz (10) using casein as substrate.

Both preparations of enterokinase exhibited a slight but detectable proteolytic activity. When enterokinase was treated with an excess of pancreatic inhibitor, 60% of this activity could be inhibited, suggesting that the preparation of enterokinase was slightly contaminated with trypsin. No definite conclusion could be drawn concerning the residual proteolytic activity of the enterokinase preparation. All further experiments were therefore corrected for the blanks containing enterokinase and casein. With the levels of enterokinase used, the values of these blanks were often identical with, and seldom significantly higher than the values for blanks containing casein only (Table II).

The hypothesis of Haanes and György(5) that enterokinase overcomes the action of trypsin inhibitor could be visualized in one of two ways. Either enterokinase binds the inhibitor, and if so, it must bind it more strongly than trypsin does, or it must partially digest the inhibitor (free or bound). In order to check the first alternative, 2 sets of determinations were performed. First trypsin-trypsin inhibitor complex was exposed to various concentrations of enterokinase at room temperature for a period of 6 hours. All tubes contained 30 γ/ml of trypsin-trypsin inhibitor complex. The amounts of enterokinase in different tubes varied as follows: 0, 10, 20, 50, 100, and 250 γ/ml. One series of tubes contained 0.1 M phosphate buffer, pH 7, and the second series contained 0.1 M acetate buffer, pH 3. The dissociation of the complex at pH 7 was 0 and at pH 3 was 38% ± 3% at all levels of enterokinase used. The second set of experiments is illustrated in Fig. 1. Addition of enterokinase slightly slowed down the formation of the complex at pH 7 and slightly accelerated the dissociation of the complex at pH 3. The observed differences are on the border line of the accuracy of the method. The results of these 2 sets of experiments rule out the possibility of preferential binding of the trypsin inhibitor by enterokinase.

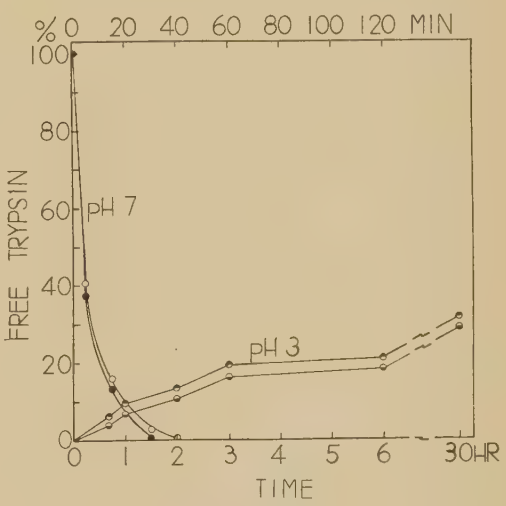

FIG. 1. Effect of time on formation and dissociation of trypsin-trypsin inhibitor complex. Upper abscissa scale in min. refers to tubes 1 and 2. Tube 1 (open circle) contained trypsin 50 γ/ml, inhibitor 25 γ/ml, 1.0 E.K.U./ml of enterokinase, 0.1 M phosphate buffer, pH 7. Tube 2 (solid circle) identical, but no enterokinase. Lower abscissa scale in hr refers to tubes 3 and 4. Tube 3 (circle, solid top) contained 30 γ/ml of trypsin-trypsin inhibitor complex, 0.6 E.K.U./ml of enterokinase, 0.1 M acetate buffer, pH 3.0. Tube 4 (circle, solid bottom) identical, but no enterokinase.

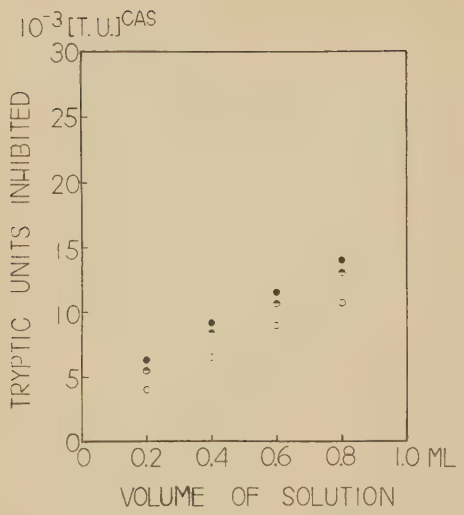

FIG. 2. Recovery of inhibitor. Tube 1 (solid circle) contained 2 γ/ml of inhibitor, 20 γ/ml (0.8 E.K.U./ml) of enterokinase. Tube 2 (open circle) contained 50 γ/ml of trypsinogen, 2 γ/ml of inhibitor. Tube 3 (circle, solid top) contained only 2 γ/ml of inhibitor. 0.1 M phosphate buffer, pH 7, in all tubes. All tubes were allowed to stand 6 hr at room temperature. Aliquots were then assayed against 25×10^{-3}[T.U.]^{cas} of trypsin.

In order to check whether enterokinase inactivated the trypsin inhibitor the experiment illustrated in Fig. 2 was performed. Comparison of values obtained with the samples from tubes 1 and 3 indicate that the inhibitor was quantitatively recovered after 6 hours exposure to the action of enterokinase. A small amount of binding was detected in tube 2, containing trypsin inhibitor and trypsinogen and was due to the partial transformation of trypsinogen into trypsin. Similar experiments in which the mixture of enterokinase and inhibitor were exposed to pH 4.5, 6.0, and 8.0 also showed a complete recovery of the inhibitor. The possibility of the inhibitor being digested by enterokinase was therefore excluded.

The results of these experiments also confirm our previous findings that when sufficient time is allowed, the presence of enterokinase does not interfere with a quantitative formation of trypsin-trypsin inhibitor complex. The above results contradict this part of the hypothesis of Haanes and György(5) which postulates that enterokinase overcomes the action of the inhibitor on trypsin.

Haanes and György(5) identified trypsin-trypsin inhibitor complex and free pancreatic trypsin inhibitor as components of the lipotropic fraction isolated by Bosshardt *et al.*(3). It then occurred to us that one of the plausible explanations of the results of Haanes and György would be to assume that in addition to the above components trypsinogen was also present. Haanes and György split the lipotropic fraction with trichloroacetic acid. They redissolved the precipitate, dialyzed it, and assayed it for proteolytic activity by the methionine method, requiring 24 hours incubation. They found that the precipitate was proteolytically active, and that addition of enterokinase did not increase this activity.

An artificial mixture containing trypsinogen (1 mg), trypsin-trypsin inhibitor complex (4 mg), and free pancreatic trypsin inhibitor (0.5 mg) was treated with trichloroacetic acid. The precipitate was redissolved. A solution of enterokinase was added to one portion, while the other contained no enterokinase but an equivalent amount of water. Aliquots were withdrawn at different time intervals. The results indicate (Table I) that the action of enterokinase can be detected only during the early periods of exposure, using the relatively rapid method of Kunitz(10). The activation of trypsinogen in the absence of

TABLE I. Effect of Enterokinase on Proteolytic Activity of a Mixture of Trypsinogen and Trypsin.

Time	E_{280} 1	2	3	4
0 min.	.208	.210	.286	.283
30	.243	.224	.337	.291
60	.252		.340	.300
90	.256		.351	.303
150		.231		
180	.252		.350	.313
5 hr		.244		
6	.258	.253	.347	.320
20	.044	.041	.352	.340
24	.006	.002	.354	.349

Tube 1 contained trichloroacetic acid precipitate dissolved in enough 0.1 M phosphate buffer, pH 7, to produce a solution having an optical density of 0.350 at 280 mμ, 0.5 E.K.U./ml, room temperature; tube 2 same as tube 1 but without enterokinase. Tube 3 contained trichloroacetic acid precipitate dissolved in enough 0.1 M acetate buffer, pH 5.6, to produce a solution having an optical density of 0.350 at 280 mμ, 0.5 E.K.U./ml, 5°C; tube 4 same as tube 3 but without enterokinase. All figures are corrected for the blanks.

TABLE II. Conversion of Trypsinogen into Trypsin in the Presence of Trypsin Inhibitor.

Tube	E_{280} 0	1 hr	2 hr	4 hr
1	.065	.057	.063	.055
2	.065	.063	.064	.063
3	.092	.270	.390	.460
Casein control	.064	.060	.062	.061

Tube 1 contained trypsinogen 50 γ/ml and inhibitor 2 γ/ml. Tube 2 contained inhibitor 2 γ/ml and enterokinase 20 γ/ml. Tube 3 contained trypsinogen 25 γ/ml, inhibitor 2 γ/ml and enterokinase 10 γ/ml. 0.1 M phosphate buffer in all tubes. 1 ml aliquots were withdrawn at indicated times.

enterokinase was accomplished by trypsin, when sufficient time was allowed. Long exposures to pH 7 resulted in inactivation of trypsin independently of the presence of enterokinase.

Haanes and György(5) recombined the trichloroacetic acid precipitate with the trichloroacetic acid soluble fraction and found no proteolytic activity. Addition of enterokinase brought about a demonstrable proteolytic activity and led the authors to conclude that enterokinase overcomes the action of inhibitor. The experiment shown in Table II indicates that trypsinogen could be activated even in presence of a considerable amount of inhibitor. The activation time is, of course, somewhat increased, which is in agreement with the previous findings of Kunitz(11).

The definite proof that the lipotropic fraction contained trypsinogen could be obtained only by direct analysis of the lipotropic fraction for trypsinogen under rigorous conditions. Unfortunately, the lipotropic fraction was not available to us. The suggested explanation remains therefore tentative. The experiments described in the present paper were made with artificial mixtures of crystalline compounds and they do not rule out a possibility that Haanes and György's preparation(5) contained some additional factor(s) responsible for the dissociation of the complex.

Summary. 1. Using highly purified systems, no evidence was found that enterokinase overcomes the action of pancreatic trypsin inhibitor on trypsin. On the contrary, the inhibitor, after being exposed to the action of enterokinase, was quantitatively recovered, indicating that enterokinase neither formed a strong complex with the inhibitor, nor digested it. 2. It is tentatively suggested that the results which led to the postulation of the new activity of enterokinase could be explained by the assumption that the lipotropic fraction contained trypsinogen in addition to trypsin-trypsin inhibitor complex and free inhibitor.

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Factors Underlying Bacterial Enzyme Synthesis.* (20125)

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Although it has long been known that *E. coli* utilizes ammonia for growth, and, therefore, synthesizes its protein requirements from this substance, the adaptive formation of formic hydrogenlyase in this organism seems only to take place in the presence of certain amino acids. Billen and Lichstein(1) have shown that the formation of this enzyme in growing cells is stimulated by several amino acids, the most important of these being L-glutamate and DL-methionine. Formation of the enzyme also takes place if resting cells are incubated in the presence of glucose and L-glutamate for 6 hours. Pinsky and Stokes(2) have pointed out that both a source of energy and certain amino acids are required for the development of the enzyme in resting cells of *E. coli*. They find that L-arginine, L-aspartic acid and L-glutamic acid are extremely important for synthesis, that L-cystine, glycine, DL-serine, and DL-threonine have stimulatory effects, whilst other amino acids including L-cysteine, L-leucine, and DL-methionine are inhibitory.

In this paper, studies are reported of the means by which energy for formic hydrogenlyase synthesis may be obtained and of the nitrogen sources used in formic hydrogenlyase synthesis by 2 strains of *E. coli*.

Methods. The strains of *E. coli* used in the present investigations were isolated from feces (Strain 1), and from pus (Strain 2). Both were maintained on nutrient agar at 37°C. Twelve to 18 hours cultures were used, substantially the same results being obtained with these as with younger (5 to 8 hour) cultures. The bacteria were scraped off the agar surface, washed 3 times in distilled water, and a bacterial suspension was made up to contain 3 mg (dry weight) per ml. The Warburg manometric apparatus was used for determination of enzymic activity. Each manometric vessel contained 0.5 ml of 0.2 *M* sodium phosphate buffer (pH 7.4, unless otherwise stated), 0.2 ml of *M* sodium formate or *M* glycerol, or 0.5 *M* glucose in a final volume of 3.0 ml and other substances as specified. 0.5 ml of the bacterial suspension was placed in the side arm, and filter paper, moistened with KOH, was inserted in the center well to absorb liberated carbon dioxide. After gassing for 15 minutes with nitrogen, and equilibration, the bacterial suspension was tipped into the main vessel. All experiments were carried out at 37°C. After the addition of the bacteria to the solutions in the main vessel, an interval of time always elapsed before the first appearance of hydrogen (indicating the presence of formic hydrogenlyase) was observed, and a further period of time was required before a constant rate of hydrogen evolution was reached. These initial periods are termed Lag T_1 and Lag T_2, respectively, and the final enzyme activity is expressed as Q_{H_2}, this being the volume of hydrogen (cmm) evolved per mg dry weight bacteria per hour. For the experiments on preadapted cells, organisms were used which had been grown overnight in a broth containing yeast extract, peptone and glucose, and which, therefore, possessed formic hydrogenlyase activity.

Results. Energy requirements for formic hydrogenlyase synthesis. Stephenson and Stickland(3) observed that formic hydrogenlyase synthesis takes place in the presence of formate and a nutrient tryptic broth. Pinsky and Stokes subsequently(2) found that such synthesis does not occur in the presence of formate and a casein hydrolysate unless glucose is also added. We have found that when adaptation is allowed to take place in the presence of sodium formate, and of bactopeptone as a source of nitrogen, a marked stimulation of the final rate of hydrogen evo-

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TABLE I. Effects of Sodium Salts of DL-Aspartate, Fumarate, and Nitrate on Hydrogen Production by *E. coli* from Formate (0.067*M*) or Glycerol (0.067*M*) in Presence of Peptone.

Strain of *E. coli*	System*	Lag, min. T_1	Lag, min. T_2	Final Q_{H_2}
1	F + 1% p	60	80	50
	F + 1% p + .1 *M* DL-aspartate	"	110	187
	F + 1% p + .1 *M* f	"	100	198
1	F + 1% p	40	80	70
	F + 1% p + .01 *M* f	"	80	368
1	F + 1% p	100	100	19
	F + 1% p + .01 *M* n	30	70	271
2	F + 1.67% p	"	90	221
	F + 1.67% p + .01 *M* f	"	70	328
	F + 1.67% p + .01 *M* n	"	40	307
1	G + 1% p	>180	—	0
	G + 1% p + .01 *M* f	160	170	52
1	1% p	>180	—	0
	1% p + .01 *M* f	> "	—	0
	1% p + .01 *M* n	> "	—	0

* F = formate; G = glycerol; p = peptone; f = fumarate; n = nitrate.

TABLE II. Effects of Sodium Fumarate, Sodium Nitrate, Ammonium Chloride and Amino Acids on the Formic Hydrogenlyase Activity of Adapted Cells.

Addition	Formic hydrogenlyase activity, % of control Strain 1	Strain 2
Nil	100	100
Dl-Serine	107	105
L-Histidine	98	100
L-Arginine	103	102
L-Leucine	103	104
DL-Aspartate	93	86
L-Lysine	—	103
DL-Valine	—	104
20 amino acids	102	103
.01 *M* Sodium fumarate	66	43
.01 *M* Sodium nitrate	58	41
.1 *M* Ammonium chloride	106	100

Amino acid concentrations are as shown in legend to Fig. 1. Enzymatic activity was estimated by measurement of the evolution of hydrogen from formate during a 20 min. period. The data in this table summarize the results of several experiments.

lution is brought about by the addition to the mixture of either DL-aspartate, fumarate or nitrate. The addition of succinate produces no effect. These effects are observed, with minor quantitative differences, with both the strains of *E. coli* that we have employed. Typical results are shown in Table I. None of these compounds stimulates the evolution of hydrogen by pre-adapted cells (Table II). Indeed, the presence of fumarate or nitrate diminishes the rate of evolution of hydrogen by these cells, probably because of their abilities to act as hydrogen acceptors. The observed stimulation on the enzymic activity of unadapted cells must presumably be due to an accelerative effect on the process of enzyme synthesis. It is known that formic hydrogenlyase formation in *E. coli* is not dependent upon cell proliferation(2,3), and turbidity measurements in some of our experiments have confirmed this fact. It was found also that the addition of nitrate to a formate-peptone medium produced a maximum effect on adaptation with less than 5% increase in turbidity. The work of Quastel *et al.*(4,5), has shown that the presence of L-aspartate, fumarate or nitrate will enable cells of *E. coli* to proliferate in the absence of oxygen. Our present results show that these substances will also serve as energy sources for enzyme synthesis in the absence of cell growth. The energy required for the increased rate of synthesis is derived from an anaerobic oxidation accomplished by fumarate or by the other hydrogen acceptors tested. It is of interest in this connection to recall the observation of Quastel and Wooldridge(6) that anaerobic growth of *E. coli* is far greater in a formate-fumarate medium than in a fumarate medium devoid of formate; the explanation advanced to account for this phenomenon was that extra energy became available through the oxidation of formate by fumarate. The effect of fumarate in enhancing the rate of synthesis of formic hydrogenlyase is also seen clearly when glycerol is present as hydrogen donor. Whilst a mixture of glycerol and peptone or a mixture of fumarate and peptone is ineffective in securing synthesis of the enzyme in *E. coli*, the addition of fumarate to a glycerol-peptone mixture brings about the synthesis (Table I).

Effects of DL-aspartate, and of ammonia, on synthesis of formic hydrogenlyase. It is likely that the presence of aspartate stimulates adaptation, owing to its conversion to fumarate in *E. coli* (Quastel and Woolf(7)). This conclusion is supported by the fact that the presence of ammonium ions markedly

TABLE III. Effect of Ammonium Chloride and Sodium Nitrate on Formic Hydrogenlyase Formation in *E. coli*.

Strain	System*	Lag, min. T_1	Lag, min. T_2	Final Q_{H_2}
1	G + 5 am†	50	80	592
	G + 5 am + .1 *M* ammonium Cl	50	80	774
	G + 5 am + .01 *M* sodium nitrate	>125	—	0
2	F + 1% p	40	90	97
	F + 1% p + .1 *M* ammonium Cl	80	80	12
1	F + 1.67% p	30	70	56
	F + 1.67% p + 0.1 *M* ammonium Cl	>120	—	0
	F + 1.67% p + .01 *M* sodium nitrate	30	70	368
	F + 1.67% p + .1 *M* ammonium Cl + .01 *M* sodium nitrate	30	70	110

* G = glucose; am = amino acids; F = formate; p = peptone.

† Dl-serine, L-histidine, L-arginine, L-leucine, and Dl-aspartate, as in Fig. 1.

affects formic hydrogenlyase synthesis. The presence of 0.1 *M* ammonium chloride strongly, or completely, inhibits formic hydrogenlyase formation in the presence of a mixture of formate and peptone but has no inhibitory effect in the presence of a mixture of glucose and amino acids (Table III). It is suggested that in a formate-peptone mixture, energy is supplied for adaptation by the transfer of hydrogen from a donor (formate or an amino acid) to fumarate, which arises by the action of aspartase from aspartate present in the peptone. The presence of ammonium ions shifts the equilibrium, L-aspartate $\rightleftarrows$ fumarate + NH_3, to the left, removing the fumarate and, therefore, inhibiting adaptation. In the presence of glucose, the energy liberated by glycolysis is available for adaptation, and the phenomenon may take place even in the presence of excess ammonium ions. Doubtless L-aspartate plays a role as hydrogen acceptor, after conversion to fumarate, even in the presence of glucose, and thus affects the rate of synthesis of the enzyme.

We have found that aspartate may be replaced by fumarate but not by succinate for formic hydrogenlyase synthesis. The possibility that the presence of L-aspartate may exert specific effects is not, of course, excluded by these results.

Effect of nitrate on formic hydrogenlyase synthesis. It is already known(2,8) that the presence of nitrate may inhibit formic hydrogenlyase synthesis, and we have found that nitrate inhibits adaptation in the presence of glucose and an amino acid mixture (Table III). Its inhibitory effects may be attributed partly to the formation of nitrite, which also inhibits adaptation(2). On the other hand, as already stated, the presence of nitrate stimulates synthesis of the enzyme in a formate-peptone medium. Probably the energy released by the oxidation of formate by nitrate leads to a stimulation, which more than compensates for the inhibitory effect of nitrate. If opposed effects of this description are involved when nitrate is present, the magnitude of each effect may vary from strain to strain, and this may explain why, in both the strains of *E. coli* with which we have worked, nitrate stimulates adaptation in the presence of excess formate, while in the experiments of Pinsky and Stokes, only inhibitory effects of nitrate are found, even in the presence of excess formate. It is of some importance that the inhibitory effects of ammonium ions on formic hydrogenlyase synthesis may be counteracted by the presence of nitrate (Table III), a fact to be anticipated if nitrate oxidation of formate, or of other substances takes place.

Amino acid requirements for formic hydrogenlyase synthesis. In the presence of glucose and a mixture of the amino acids found in casein hydrolysate, both Strain 1 and Strain 2 show almost identical lag periods and final rates of hydrogen evolution. A marked difference, however, is observed between the 2 strains, in their specific amino acid requirements for formic hydrogenlyase synthesis. The effect on formic hydrogenlyase synthesis of omitting each amino acid from a mixture of the amino acids of a casein hydrolysate has been investigated with the following results. The amino acids, whose omission brings about a significant increase in the lag period and a decrease in final enzymatic activity are, for Strain 1, DL-serine, L-histidine, L-leucine, and DL-aspartate; and for Strain 2, DL-serine, L-histidine, L-arginine, L-leucine, DL-

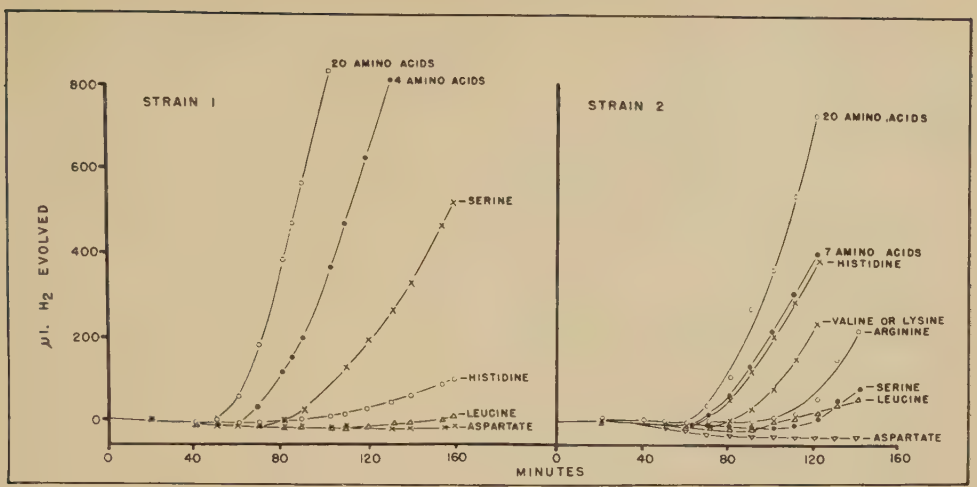

FIG. 1. Effects of amino acids on formic hydrogenlyase synthesis. Glucose (0.033*M*) present in all vessels. Amino acids present in same amount per vessel as in 1 ml of a 5% casein hydrolysate (according to data of Williamson(9)), with addition of 0.75 mg/vessel of DL-norleucine. Where only DL form of the amino acid was available, amino acid was added which gave the appropriate concentration of the L-form. Composition of 4-amino acid mixture in each vessel (for Strain 1) was as follows: DL-serine, 1.67 mg/ml; L-histidine, 0.33 mg/ml; DL-aspartic acid, 1.4 mg/ml; L-leucine, 2.4 mg/ml. Composition of 7-amino acid mixture in each vessel (for Strain 2) was as follows: amino acids as above together with L-arginine, 0.65 mg/ml; L-lysine, 1.0 mg/ml; DL-valine, 1.77 mg/ml.

aspartate, L-lysine, and DL-valine. It is found that for each strain a mixture of amino acids, as specified above, can almost replace a mixture of all the amino acids in a casein hydrolysate as a means of promoting adaptation. Results illustrating this fact and the effect of omission of each amino acid from the simplified mixtures are shown in Fig. 1.

DL-serine, L-leucine, and DL-aspartate are of considerable importance for the enzyme synthesis in both strains. The presence of L-histidine greatly aids the production of the enzyme in Strain 1, but apparently has little effect in Strain 2. L-arginine is of importance for Strain 2, but has no effect on enzyme production in Strain 1. As the results reported in Table II show, none of these amino acids, nor a mixture of 20 present in a casein hydrolysate, has any effect on the formic hydrogenlyase activity of adapted cells. These findings on amino acid requirements agree with those reported by Pinsky and Stokes(2) in showing the importance of DL-serine and L-aspartic acid, and, in one strain, of L-arginine, for formic hydrogenlyase formation. With both our strains of *E. coli* L-glutamate alone can serve as a nitrogen source for formic hydrogenlyase synthesis in the presence of glucose. It seems to be dispensable, however, in the presence of a mixture of amino acids and glucose. When L-glutamate alone is present in a glucose medium, the lag period before the appearance of enzymatic activity is almost twice as long as when an amino acid mixture is used, and the final activity is much lower.

It is possible that variations in amino acid requirements for formic hydrogenlyase formation, between the different strains of *E. coli*, may be due to differences between the rates of amino acid interconversion in these strains.

Summary. 1. A marked stimulation of the synthesis of formic hydrogenlyase by *E. coli* in the presence of sodium formate and peptone is brought about by addition of either DL-aspartate, fumarate, or nitrate under conditions when little or no proliferation takes place. Addition of succinate produces no effect. None of these compounds stimulates activity of the enzyme in pre-adapted cells. Whilst a mixture of glycerol and peptone, or a mixture of fumarate and peptone does not secure enzyme synthesis, a mixture of glycerol, fumarate and peptone is effective. The energy for synthesis in resting cells may thus

be obtained by the interaction of suitable hydrogen donors and hydrogen acceptors. Aspartate is effective presumably because it is converted by aspartase into fumarate. This conclusion is supported by the fact that the presence of ammonium ions inhibits adaptation in a formate-peptone medium, but not in a glucose-*amino acid* medium where energy is secured by glucose breakdown. 2. Certain amino acids are required for the enzyme synthesis. The most effective are leucine, aspartate, histidine, and serine. Requirements for specific amino acids appear to vary with the strain of the organism.

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Toxicity of Non-Ionic Detergents for the Chick Embryo.* (20126)

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A recent investigation by Morton *et al.*(1) showed that the non-ionic detergent Tween 80 in a concentration of 0.5 mg/ml of medium inhibited the growth of fibroblasts in tissue cultures while in adult rats Tween 80 required a concentration of 5-10 g/kg of body weight(2) to be toxic. The higher toxicity of the detergent in tissue cultures raised the question as to whether rapidly growing and less differentiated cells are more sensitive to the detergent than the non-proliferating mature cells. A possibility of comparing the sensitivity to non-ionic detergents of cells in different degrees of proliferative activity and differentiation seemed to be present in the developing chick embryo. Whether non-ionic detergents had a teratogenic effect owing to their surface activity was also of interest because of the great importance of the interaction of cell surfaces during early embryonic development(3). Also, substantial amounts of non-ionic detergents such as Tween 80 are contained in various vitamin preparations which are given to premature infants as well as to mature ones. Therefore, from a clinical point of view, toxicity tests on immature tissue had some interest. Since qualitative and quantitative differences exist in the action of different non-ionic detergents upon different biological systems, such as the tubercle bacillus(4) or the red blood cell(5), it seemed desirable to compare at least two detergents of this type in their effect upon the chick embryo. We chose Tween 80† and Triton X-100† for this comparison.

Materials and methods. Tween 80 is described in the literature as polyoxethylene sorbitan mono-oleate and Triton X-100 as arylalkyl polyether of phenol. The structural formulae are given in the paper by Dubos quoted above(4). Solutions of these substances were made up in distilled water to give the desired amount of material in 0.1 ml. To reach the highest concentrations of Tween 80 the volume was increased to 0.5 ml. The control eggs were injected with 0.6% NaCl solution. All solutions were sterilized by auto-

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† We are indebted to Atlas Powder Co. for the samples of Tween 80 and to Rohm and Haas for the samples of Triton X-100.

TABLE I. Toxicity of Triton X-100 for Chick Embryos at Different Stages of Development.

Age (days incubation)	Amt of Triton inj. mg/egg	Amt of Triton inj. mg/g tissue	Total eggs inj.	% surviving
Before incubation (inj. into yolk)	.0	.0	213	67
	.1	.005	87	30
	.25	.012	194	42
7-8 days (inj. onto membrane)	.0	.0	114	82
	.12	.048	54	80
	.25	.1	59	53
	.5	.2	54	17
15-16 days (inj. onto membrane)	.0	.0	112	95
	.25	.018	20	95
	1.0	.071	54	92
	3.0	.21	23	61
	4.0	.29	45	55
	10.0	.71	24	0

claving. In most instances the solutions were injected onto the chorioallantoic membrane through a small drill hole in the eggshell which was subsequently sealed with Scotch tape. One series of experiments is listed in which the solution was injected into the yolk. The eggs were opened 3 days after injection onto the chorioallantoic membrane and 8 days after injection into the yolk. The embryos were inspected for the more obvious malformations of the head and skeleton. In view of the uncertainty of the distribution of the injected substances between yolk and embryonic tissue, our data are calculated as per whole egg or per gram of tissue of embryo plus membranes. Depending upon the calculation, the absolute values for the observed toxicities will differ. For an evaluation of the relative toxicities of the detergents which was the primary aim of the present experiments, the different calculations gave essentially the same results.

Results. Triton X-100. A dose of 0.1 mg/egg of Triton X-100 injected into the yolk previous to incubation was toxic. 67% of the controls survived, while only 30% and 42% of the experimental embryos survived the same period after injection of 0.1 and 0.25 mg of the detergent, respectively. The toxicity increased slowly with the dosage and an injection of 2.5 mg of Triton per egg reduced the number of survivors to 18%. Assuming an equal distribution of the injected compound between yolk and embryo the minimum toxic amount of 0.1 mg/egg would correspond to a concentration of about 0.005 mg/g of embryo based on the weight for the yolk plus the embryo of about 20 g on the eighth day of incubation.

In experiments in which Triton was injected onto the chorioallantoic membrane, we assume that most of the substance is taken up by the embryonic circulation. Therefore, calculations are based on the total weight of the embryo plus membranes. On the 7th-8th day of incubation 0.05 mg/g Triton had no toxic effect, 0.1 mg/g had a slight effect, and 0.2 mg/g reduced the number of survivors from 82% in the controls to 17% in the experimental embryos. On the 15th-16th day of incubation a concentration of .07 mg/g of embryo had no toxic effect, 0.2-0.3 mg/g embryo decreased the number of survivors from 95% to 61 and 55%, respectively, and 0.71 mg/g killed all 25 embryos injected (Table I). In an experiment with 24 chickens freshly hatched to one-day-old and intraperitoneal injection of 0.5 mg/g of chick killed about one-half of the chickens within 2 hours.

Tween 80. In a large series of experiments it was attempted to demonstrate toxic effects of Tween 80 on the developing embryo after injection into the yolk prior to incubation similar to the experiments with Triton X-100. However, with this technic no toxic effects could be obtained with any consistency even after injection of very large amounts (20 mg/egg). The inadequacy of this procedure could be demonstrated in experiments with embryos after 4 days of incubation. Table II shows that after injection into the yolk of 10 mg of Tween/egg the number of survivors was almost as high as in the controls. After

TABLE II. Comparison of Toxicity of Tween 80 after Injection into Yolk and onto Membrane. Age of embryos: 4 days of incubation. Amount of Tween 80: 10 mg/egg.

	Total eggs inj.	% surviving
Saline inj. onto m*	35	71
Tween 80 inj. onto m	36	33
Saline inj. into y	36	67
Tween 80 inj. into y	36	59

* m = membrane; y = yolk.

TABLE III. Toxicity of Tween 80 for the Chick Embryo at Different Stages of Development.

Age (days incubation)	Amt Tween inj. mg/egg	Amt Tween inj. mg/g tissue	Total eggs inj.	% surviving
4	.0	.0	156	79
	.25	.63	26	85
	1.0	2.5	67	19
	3.0	7.5	32	0
	10.0	25.0	85	22
7	.0	.0	374	68
	2.5	1.0	152	53
	5.0	2.0	46	54
	10.0	4.0	103	17
15-16	.0	.0	136	98
	10.0	.7	24	96
	40.0	2.9	24	92
	100.0	7.2	32	94
	200.0	14.3	49	39

injection of the same amount of Tween 80 onto the membranes, the number of surviving embryos was decreased to 33%. The negative results after injection of Tween 80 into the yolk could be due to hydrolysis of the detergent by a lipase which is known to be present in the yolk. The smallest dose of Tween 80 which caused a decrease in the number of surviving embryos when injected onto the chorioallantoic membrane at 4 days of incubation was found to be 2 mg/g of tissue. At that early stage the membranes weigh about 300-400 mg and the weight of the embryo is reported to be 50 mg.‡ At 7-8 days of incubation toxicity is still observed with 1.5-2.5 mg while at 15-16 days of incubation about 15 mg/g of embryonic tissue are required to produce even a slight toxic effect (Table III).

Discussion. Our figures give a toxicity of Triton X-100 about 20 times greater than that of Tween 80. The surface activities of the 2 compounds are: 30 dynes per cm for Triton X-100 and 41 dynes per cm for Tween 80 at a concentration of 0.1%. Triton exhibiting a lower surface tension than Tween 80, also exhibits a greater toxicity. However, it is unlikely that this difference in toxicity is simply a function of the surface activity of these substances. Sodium oleate, which has a higher surface activity than Triton, is definitely less toxic for the 7-day-old chick embryo.

In the introduction we raised the question of a dependence of the toxicity upon the age of the embryo. The fact that higher concentrations of Tween 80 and Triton X-100 are required by the 16th day of development is of interest in this connection since by this time a far-reaching maturation of many tissues has taken place. This may mean that the more mature tissue is less sensitive to the detergent. At the same time it has to be kept in mind that in the course of this maturation detoxification mechanisms may at least in part become responsible for the apparently decreased toxicity. However, our results may be suggestive enough to make it seem advisable to test other compounds of the same class of substances in order to find out whether some of them will display larger differences in their toxicity for immature and mature tissues.

In the course of experiments no occurrence of developmental abnormalities was observed and no clue has been found as to the mechanism of the toxic action of the detergents.

Concerning the question of a possible toxicity of non-ionic detergents for premature infants one has to consider that in these babies even the maximum figure for the dietary intake is at most one-tenth of the dose found lethal in chick embryos. Studies carried out by the producers of Tween 80 indicate very good tolerance of Tween 80 in men. Hospital records of premature babies with and without Tween 80 supplement in the diet have not revealed obvious indications of ill effects. However, as long as a systematic survey is missing in regard to premature tolerance of Tween 80, observations of a toxicity for embryonic tissues, even in an unrelated species, may make it advisable to follow critically the

‡ This figure is taken from R. Rugh, *Exp. Embryology,* 1948, p.450.

history of premature infants with large Tween 80 supplements in the diet.

Summary. Two non-ionic detergents, Tween 80 and Triton X-100, have been compared with respect to their toxicity to chick embryos. Tween 80 was found to be 20 times less toxic than Triton X-100. The possibility is considered that with the increasing age of the embryos there is a decrease in the toxicity of both detergents.

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Effect of Repeated Doses of External and Internal Irradiation on Structure of the Spleen.* (20127)

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It has been pointed out(1) that the spleen is particularly adapted to a study of susceptibility of various cells to irradiation. This statement can be further extended by indicating that pathological findings in the irradiated spleen are suitable to illustrate the fate not only of cellular elements, but also of the structure of a parenchymatous organ, containing numerous free cells of various kinds in a framework of connective tissue, as a whole. For this purpose, a larger amplitude of changes could be produced by using for internal irradiation colloidal radioactive gold Au^{198}† which is short living (half life of 2.7 days), beta emitter and which can be deposited, owing to its colloidal structure(2) into the spleen, in graded amounts. This method, alone or in combination with external irradiation, enabled us to obtain in experimental animals a larger variety of changes in the spleen structure than those elicited by external irradiation alone(3) as is shown by the data presented below.

Material and methods. (a) *Animals.* Mice of C3H strain,‡ of 24 to 27 g weight, mostly males, were used throughout in these experiments. For internal irradiation they received injections of a radioisotope intraperitoneally, intrapleurally (the technic of this injection was described elsewhere(4)), intravenously or subcutaneously. For external irradiation, groups of not more than 12 mice were immobilized in horizontal position between 2 cardboard sheets inside a small box adjustable to irradiating appliance. (b) *Internal irradiation.* Colloidal Au^{198}† was injected in a volume of 0.5 cc containing 0.01 to 0.03 millicurie (small doses) or 2 millicuries (large dose). (c) *External irradiation.* Doses of 100 to 1200 r of filtered rays (Filter: 1.2 mm Sn; 0.25 mm Cu; 1.0 mm Al; TSD 40 cm; HVL 3.27 mm Cu; 250 PKV) were used. (c) *Autopsies.* Preliminary research provided information about time interval between exposure and effect, for each type and amount of irradiation, thus indicating the suitable interval between exposure and autopsy in the main experiments.

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† Radioactive Au^{198} was obtained through Dr. D. L. Tabern, Abbott Research Laboratories, North Chicago, Ill.

‡ The mice were obtained from Roscoe Jackson Memorial Laboratories, Bar Harbor, Me.

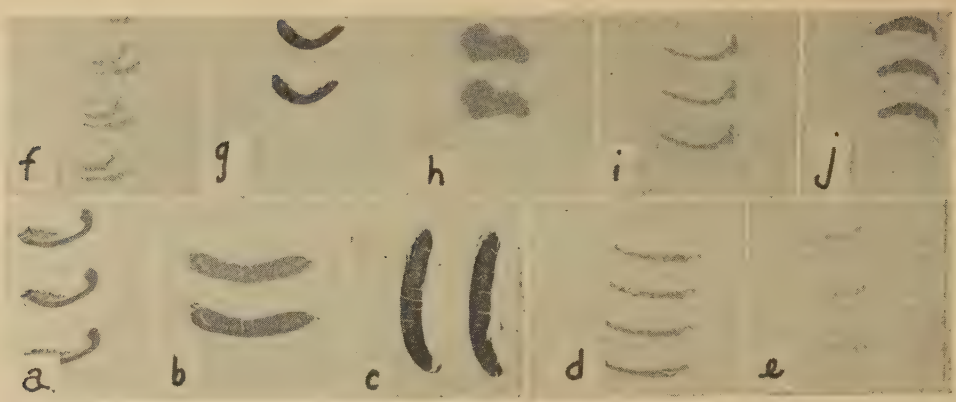

FIG. 1. Changes in size of the spleen of the mouse after irradiation (natural size of stained sections). (a) normal spleen; (b) five days after inj. of 0.01 mc of radiogold colloid H + E; (c) same, Mallory; (d) three days after inj. of 2 mc of radiogold colloid; (e) nine days after inj. of 0.01 mc and 3 days after subsequent inj. of 2 mc of radiogold, H + E; (f) three days after whole body exposure to 1200 r, H + E; (g) Nine days after inj. of 0.01 mc of radiogold and 3 days after exposure to 1200 r; (h) three days after 2 exposures to 400 r at interval of 7 days, H + E; (i) three days after 8 exposures to 100 r at intervals of 3 or 4 days; (j) three days after 4 exposures to 200 r at intervals of 1 day.

The spleens were stained with hematoxylin-eosin or by Mallory technic.

Results. I. *Effect of a small dose, single or repeated.* A. *Internal irradiation.* Four days after intrapleural or intraperitoneal injection of 0.01 to 0.05 mc of colloidal Au^{198}, the spleen was found 5 to 8 times larger (Fig. 1, b and c) as compared with an average normal spleen (Fig. 1a). Microscopic examination revealed high congestion of the organ with marked distention of connective tissue framework and of the capsule. However, lymphocytopoietic centers were conspicuous among the pulpal elements rarified by congestion of the organ. The congestion disappeared as a rule within 7 to 14 days leaving the spleen slightly enlarged and impregnated with deposit of iron pigment. Repetition of same doses of radiogold at any intervals would not increase the size of the spleen and its congestion beyond the effect of a single dose, but it would decrease the number of lymphocytic elements. B. *External irradiation.* Doses of 50 to 250 r did not induce any noticeable change in the size of the spleen. Slight increase (less than twice the size of an average spleen) was noted within 4 days after irradiation with 150 to 400 r. After repeated doses of 200 or 400 r sufficiently spaced the spleen showed enlargement by congestion (Fig. 1, h and j), while after frequent application of even smaller doses, it was shrunken (Fig. 1, i). These experiments with various small doses of x-rays variously spaced will be reported separately(5).

II. *Effect of a high irradiation dose.* A. *Internal irradiation.* A dose of 2 mc of radiogold colloid was lethal for all mice within 5 days. In sacrificed moribund animals the spleen size was ½ or ⅓ of an average normal spleen, the shrinkage being most marked in both poles (Fig. 1, d). The tissue sections presented microscopical evidence of shrinkage—contraction of connective tissue framework and separation of the capsule from the organ surface—and of congestion. Both changes were diffusely marked in the red pulp and on the periphery of the spleen, while the white pulp containing lymphocytes with pyknotic nuclei was apparently "condensed" by the shrinkage of the whole organ. B. *External irradiation.* Exposure to 1200 r was followed by the death of all mice on the 4th day with few exceptions surviving until the 5th day. At autopsy, the spleen was about 1/5 or 1/6 of the average size of the normal spleen. The shrinkage was manifested by constriction of the body of the spleen (Fig. 1, f) and microscopically by marked outlines of connective tissue bundles and by separation

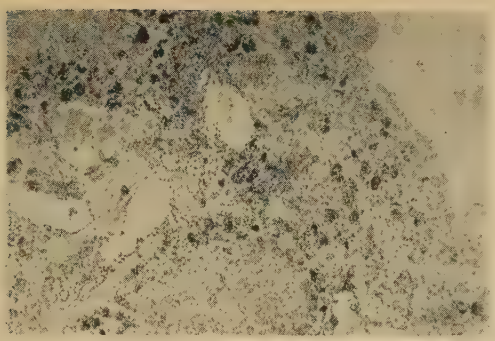

FIG. 2. Spleen of the mouse, 9 days after inj. of 0.01 mc and 3 days after subsequent inj. of 2 mc of colloidal radiogold. H + E × 50.

of the capsule from organ surface. The congestion was not marked and lymphocytic elements appeared considerably reduced in number throughout the area of the section.

III. *Small irradiation dose followed by a large dose.* (A) *Small dose internal irradiation followed by a large dose.* This method of irradiation is a combination of IA and IIA, *i.e.*, 1) the injection of 0.01 mc of radiogold colloid which was shown to induce spleen enlargement by congestion followed after 5 or 6 days by 2) the injection of 2 mc, which alone caused shrinkage and partial atrophy of the spleen. Autopsies performed on the 4th day after the 2nd injection revealed the striking effect of this combined treatment; within 96 hours the size of the spleen was reduced more than 10 times (from that shown in Fig. 1, b and c, to that of Fig. 1, e). Moreover, already the gross examination of stained spleen sections (same figure) revealed the disappearance of the pulp within the shrunken framework of the organ. This was confirmed by microscopic examination (Fig. 2), which showed nearly complete disappearance of all cellular elements with the exception of fibrous tissue and of numerous macrophages loaded with gold particles and iron pigment. Briefly, this phenomenon can be described as complete spleen atrophy induced in a mouse within 8 or 9 days by successive injection of a small and a large dose of colloidal radiogold. B. *Small dose of internal irradiation, followed by a large dose of external irradiation.* In this experiment mice pretreated with a small dose (0.01 mc) of radiogold, as above, received on the 5th or 6th day a large dose of external irradiation (1200 r) instead of radiogold. The autopsy on the 4th day after the 2nd treatment indicated that the spleen, which was highly congested after the injection of small Au^{198} dose, returned to approximately its normal size (Fig. 1, g) and did not shrink beyond it, as it was recorded above after reinjection of radiogold (2 mc) in pretreated mice. Microscopically the diffuse and extensive atrophy of the pulp was less advanced than in the above experiment (Fig. 2) and there was a marked thickening of fibrous tissue bundles in the organ framework and in its capsule. Thus, the pretreatment of a mouse with a small dose of radiogold did increase the severity of pulp destruction and framework shrinkage by external irradiation, but not so dramatically as in the above experiment.

Several series of mice were exposed to a small amount of external irradiation (100 and 250 r) and after 5 days injected with 2 mc radiogold or exposed to 1200 r. In each series the effect of combined treatment on the spleen was not significantly different from the effect of the large dose of irradiation alone. Therefore, a small dose of external irradiation had no or only a slight preparative effect on the results of subsequent treatment with massive irradiation.

Discussion. Experimental work in mice on histopathology of spleen irradiation has been reviewed by Dunlop(6) and by Murray(1). It is the consensus of opinion that after external irradiation the spleen undergoes brief early swelling and then shrinks rapidly. The initial congestion was rapidly transient; for instance, after irradiation of the mouse with 80 r some changes were found in the spleen after 4 hours, but none after 24 hours(1). The effect of 100 to 300 r was only slightly more marked. After exposure to 600 r or over, the organ was found shrunken(1) and fibrotic(6), with enormous number of macrophages.

Internal irradiation with a soluble radioisotope—$Strontium^{89}$—did not produce even in high doses (0.036 mc per g weight) any significant changes in the spleen(1). However, relatively small doses (0.23 to 0.26 mc) of a particulate radioactive material—chromic

phosphate with half life of 14.3 days—"reduced within 15 to 23 days the spleen almost to a connective tissue framework and macrophages"(7). This difference in the effect of soluble and of particulate radioisotopes depends obviously on the retention of the latter in the spleen. The particulate radioisotope used in our experiments—colloidal Au^{198} with half life of 2.7 days—was found to be suitable to produce graded changes in the structure of the spleen. This advantage can be accounted by shorter half life and smaller particles of colloidal radiogold.

The high congestion induced in the spleen by minimal doses (0.01 to 0.05 mc) of colloidal Au^{198} was, obviously analogous to the initial effect of external irradiation, but more marked and more lasting, owing to protracted action of deposited radioactive colloid on tissues.

The rapid shrinkage of the spleen after injection of high doses of the same isotope was similar to the effect of high doses of external irradiation. As it would be expected, numerous radioactive particles diffusely spread throughout the tissue produced more severe and more extensive changes in the spleen structure than higher, but short acting doses of external irradiation.

It was found unexpectedly that successive injections at a very short time interval, of a minimal dose (0.01 mc) and a 200 times larger dose (2.0 mc) of colloidal radiogold induced rapidly a complete atrophy of the spleen, leaving only the connective tissue framework and the pigment and gold loaded macrophages. Obviously, a minimal dose of radiogold incapable by itself to induce significant regressive changes in the spleen had some preparatory action on the organ by rendering its tissues more susceptible to the subsequent blow inflicted by a high dose of radiogold. There is some evidence in the work of several authors (6) that the radiosensitivity of the spleen increases with increase of the blood supply. It can be presumed therefore that the preparative effect of the small dose of radiogold is due to its remarkable lasting congestive effect on the spleen. This interpretation will agree with our finding that small doses (50 to 200 r) of external irradiation which induce only slight and transient congestion of the spleen or none did not exert "preparative" effect on the spleen treated subsequently with high doses of radiogold or of x-rays. It may be concluded that high congestion of the spleen induced by a small dose of colloidal radiogold constituted "the preparative factor" responsible for rapidly progressing severe atrophy of the spleen after injection of a high dose of radiogold into the same mouse.

It should be emphasized that both the high congestion and the advanced atrophy of the spleen were induced regularly only in one strain of mice—C3H and by intrapleural or intraperitoneal injection. It may be presumed that these findings can be reproduced in other animal species and by other routes (intravenously) by varying the dosage, the interval between injections and perhaps some other conditions of the experiment.

Summary and conclusions. 1. Minimal doses of internal irradiation (0.01 to 0.05 mc of colloidal radiogold) induced in C3H mice within 5 or 6 days a very marked congestive swelling of the spleen which lasted for about 14 days. No significant effect on size of the spleen could be obtained within a wide range (50 to 200 r) of small doses of external irradiation. 2. Large doses of radiogold (2 mc) as well as of external irradiation (1200 r) caused shrinkage and partial atrophy of the spleen within 3 days, the effect of the former being more severe. 3. Large dose of colloidal radiogold (2 mc) injected 5 or 6 days after pretreatment with a minimal dose (0.01 mc) of the same radioisotope induced within 3 days more marked shrinkage and more complete atrophy of the spleen than the same dose of radiogold without pretreatment. Substitution of radiogold (either of a preparative dose or of a large dose) by analogous doses of external irradiation did not reproduce the same drastic changes of the spleen. 4. These findings were interpreted in the light of the existing evidence that the radiosensitivity of the spleen increases with the increase of the blood supply. It was presumed that high congestion of the spleen induced by a small dose of colloidal radiogold constituted "the preparative factor" responsible for rapidly progressive severe atrophy of the spleen after subsequent

injection of a high dose of radiogold into the same mouse.

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In vitro and *In vivo* Activity of Candicidin on Pathogenic Fungi. (20128)

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Lechevalier, Acker, Corke, Haenseler, and Waksman(1) have established that candicidin is an antifungal agent with activity against certain filamentous and yeast-like fungi. The purpose of the present communication is to define the antifungal spectrum of the water-soluble fraction of candicidin against pathogenic fungi and to estimate its protective effect in mice with systemic mycoses.

Materials and methods. The antibiotic was dissolved in various concentrations in an agar medium containing 2% glucose and 1% neopeptone adjusted to pH 7. A 1:1000 volumetric suspension of the various fungi were streaked over the agar surface. The plates were incubated at 37°C and at room temperature. Estimation of growth inhibition was made within 2 to 3 days after growth was evident in the control plates. This was 2 to 3 days after incubation for rapidly growing yeast-like fungi, such as *Cryptococcus neoformans* and *Candida albicans,* and 7 to 8 days for slower-growing fungi such as *Histoplasma capsulatum* and *Coccidioides immitis.* To determine fungicidal activity a suspension of *C. albicans* in brain heart infusion broth containing approximately one million cells/ml was prepared. The antibiotic was then added in concentrations of 10 and 50 μg/ml. At 2, 4, 6, 8, and 24 hours samples of the broth were removed and the quantity of viable cells determined by plate counts on glucose peptone medium. The heart infusion broth was maintained at 37°C during the test. The final concentration of antibiotic in the media on which the colony counts were made was not sufficient to exert a fungistatic effect.

Experimental infections. Throughout, 20 g white, male mice were used. In some cases injections of the fungi were made intraperitoneally and, in others, intravenously by way of the tail vein. The quantity of injected cells was appropriately regulated so as to avoid overwhelming infection and rapid death. The aim for the most part was to produce sublethal infections of sufficient severity to enable gross comparisons when the animals were sacrificed. The candicidin was administered intraperitoneally in all instances. Generally, it was given for 10 successive doses beginning on the day of infection. The animals were sacrificed on the 14th day.

Results. In vitro antifungal activity. Preliminary studies showed that the antibiotic activity was considerably reduced under acid conditions. At pH 5 and pH 6 approximately 100 times more antibiotic was required for inhibition than at pH 7 and pH 8, in which range the activity was maximal. Human blood slightly diminished the fungistatic power of candicidin. The antifungal activity was slightly greater at room temperature than at 37°C. Comparable results were obtained with broth and with agar streak methods. The antibiotic activity was maintained undiminished in brain heart infusion broth incubated at

TABLE I. Sensitivity of *Candida* Species to Candicidin.

Organism	Strain	Min. conc. for complete inhibition (μg/ml)
C. albicans	12A2	.5
	3	2.5
	4	.5
	5	5
	6	5
C. albicans stellatoidea	12B1	2
	2	5
C. tropicalis	12C1	5
	2	10
	3	2.5
C. pseudotropicalis	12D1	2.5
	2	2.5
C. guilliermondi	12E1	2.5
	2	5
	3	2.5
C. krusei	12F1	5
	2	10
C. parakrusei	12G1	50
	2	25
	3	50

TABLE II. Sensitivity of Pathogenic Fungi to Candicidin.

Organism	Strain	Min. conc. for complete inhibition (μg/ml)
*Blastomyces dermatitidis**	5A2	.5
" " *	5A3	1
*Histoplasma capsulatum**	7A6	1
" " *	7A5	.5
*Paracoccidioides brasiliensis**	8A7	5
Coccidioides immitis	9A4	500
" "	9A5	500
Cryptococcus neoformans	15A2	1
" "	15A5	2.5
*Sporotrichum schenckii**	6A3	200
" " *	6A4	100
Nocardia asteroides	19A1	200
Geotrichum spp.	13A1	2.5
Hormodendrum pedrosoi	4A1	10
Trichophyton mentagrophytes	2A1	500
" *tonsurans*	2C1	500
" *rubrum*	2B1	500
Microsporum canis	1B1	500
" *audouini*	1A1	500
Epidermophyton floccosum	3A2	500

* Yeast phase.

37°C for one week before testing. The drug was thus stable for this period of time.

The sensitivity of various species of *Candida* is shown in Table I. *Candida albicans,* the chief pathogen in this group, was very sensitive. Strain variations were evident. *Candida parakrusei,* which is but rarely a human pathogen, was the most insensitive of this group.

The activity of candicidin against the major pathogenic species is shown in Table II. A unique feature of the antifungal spectrum is the resistance of *Coccidioides immitis* and the ringworm fungi (species of *Trichophyton, Microsporum,* and *Epidermophyton*). This parallels the experience with actidione, another antifungal antibiotic(2). The actinomycete, *Nocardia asteroides,* was resistant. Exclusive of these fungi, candicidin was markedly inhibitory for the major fungus pathogens. When the same plates were examined 2 weeks after the initial reading, there was usually some growth at the minimal concentration which had originally been inhibitory on the first reading. This suggested some degree of deterioration of the antibiotic.

Fungicidal activity. With both 10 and 50 μg of candicidin/ml of the brain heart infusion broth, the *C. albicans* cell count was reduced approximately 45% in 2 hours, 60% in 4 hours, and 90% in 6 hours. Approximately 1% of the cells were viable in 24 hours. The initial colony counts were made on the 2nd day after plating. On the 7th day many more colonies were evident in the same plates. Thus, many of the cells were simply inhibited and not killed.

Toxicity. The LD_{50} for 20 g Carworth Farm white, male mice ranged between 50 and 65 mg/kilo for different lots of candicidin. The maximum tolerated daily intraperitoneal dose for a period of 14 days was 0.8 mg/mouse (40 mg/kilo). This figure, too, varied for different lots. No irritation was caused by 0.3 ml of a 1% solution placed into the conjunctival sac of rabbits. The 1% solution was not irritating to the human oral mucosa when swabbed over the tongue for one minute every 3 hours for 2 days during the day time. Injection of the 1% solution intradermally and subcutaneously into mice and guinea pigs caused necrosis within 24 hours with the subsequent development of a severe slough. The 0.1% solution was less toxic when injected into the skin of these animals, but moderate necrosis still developed.

Protective effect in experimental infections. Candicidin exerted a marked suppressive effect

TABLE III. Effect of Candicidin on Moniliasis and Torulosis in Mice.

Organism	Strain	No. mice	Infective dose	Treatment	Mortality	Positive* cultures (survivors)	Gross† findings (survivors)	Smears‡
Candida albicans	12A2	46	0.3 ml of 1:50000 suspension intrav.	0.75 mg Candicidin intraper. daily, 10 days beginning day of infection	3/46	1/43	0/43	—
"	3	48			2/48	0/46	0/46	—
"	2	24		0	8/24	16/16	16/16	—
"	3	24		0	6/24	18/18	18/18	—
Cryptococcus neoformans	15A3	18	0.3 ml of 1:1000 suspension intrav.	0.75 mg Candicidin intraper. daily, 10 days beginning day of infection	4/18	14/14	—	14/14(++)
"	5	24	0.3 ml of 1:10000 suspension intrav.		2/24	22/22	—	22/22(+)
"	3	18	0.3 ml of 1:1000 suspension intrav.	0	2/18	15/15	—	15/15(++++)
"	5	12	0.2 ml of 1:10000 suspension intrav.	0	0/12	12/12	—	12/12(++++)

* Cultures prepared from kidneys of mice with *C. albicans* and from brains of animals inoculated with *C. neoformans*. Animals sacrificed 14th day.

† Gross findings in mouse moniliasis refers to presence of grossly visible kidney lesions.

‡ Smears are India ink preparations made from brains of animals inoculated with *C. neoformans*. The No. of (+) marks refers to relative quantity of capsulated cells seen in smears. The latter are prepared by emulsifying a portion of brain tissue in a few drops of India ink.

on moniliasis in mice, as shown in Table III. The untreated mice exhibited enlarged kidneys with irregular paleness of the surface or with discrete pinpoint white surface lesions. The kidneys of treated mice were rendered sterile and the kidneys grossly were normal. In another experiment not recorded in Table III, the administration of candicidin was delayed until the 4th day after intravenous inoculation in order to allow the infection to become well established. It was then given daily at the rate of 0.75 mg/ day until the 14th day, at which time the sacrificed animals did not show gross lesions, although positive kidney cultures were obtained from 3 of 22 mice. This indicated that candicidin was curative in mouse moniliasis. Candicidin did not have so striking an effect of torulosis of mice (Table III) as it did in moniliasis. Cultures of the brains of treated animals were always positive. Definite suppression was evident, however, as indicated by the diminished number of organisms present in India ink brain smears. When lethal doses of *C. neoformans* were given intravenously, candicidin was not effective in delaying the day of death or in altering the mortality rate. Candicidin exerted a *marked protective effect* on blastomycosis in mice (Table IV). The untreated animals grossly showed numerous granulomatous lesions in their lungs although there was no evidence of infection in the treated group. For the most part the lungs of treated animals were completely free of the organism as indicated by negative cultures. Strain 5A2, which is highly virulent, produced 100% mortality in untreated mice under the conditions of the experiment. Treatment with candicidin prevented this.

Candicidin had a significant protective effect on mice inoculated intraperitoneally with *Histoplasma capsulatum* (Table IV). Strain 7A6 killed 14 of 16 untreated mice in 14 days, whereas only 4 of 24 treated mice died in the same period. Untreated mice which survived showed enlarged spleens. Splenomegaly was present in only a few of the treated mice. Cultures of the spleens of treated animals were positive in about 75% of the survivors, although in most cases only a few colonies were evident as compared to

TABLE IV. Effect of Candicidin on Blastomycosis and Histoplasmosis in Mice.

Organism	Strain	No. mice	Infective dose	Treatment	Mortality	Positive* cultures (survivors)	Gross† findings (survivors)
Blastomyces dermatitidis	5A2	24	0.3 ml of 1:10000 suspension intrav.	0.75 mg Candicidin intraper. daily, 10 days beginning day of infection	0/24	3/24	0/24
"	3	30			2/30	2/28	0/28
"	2	18		0	18/18	—	—
"	3	12		0	6/12	5/5	6/6
Histoplasma capsulatum	7A6	24	0.5 ml of 1:100 susp. in 5% gastric mucin intraper.	0.75 mg Candicidin intraper. daily, 10 days beginning day of infection	4/24	16/20	2/20
"	8	18			2/18	12/16	3/16
"	6	16		0	14/16	2/2	2/2
"	8	18		0	1/18	15/15	15/15

* Cultures were prepared from lungs of animals with blastomycosis and from spleens of mice with histoplasmosis. Animals sacrificed 14th day.
† Gross findings in mouse blastomycosis refers to grossly visible nodules in lungs, and in mouse histoplasmosis to enlarged spleens.

large numbers recovered from the spleens of untreated animals. When 0.3 ml of 1:100 *H. capsulatum* suspension was inoculated intravenously into 12 mice and treated according to the same dosage schedule, the protective effect of candicidin was not impressive. The spleens of treated animals were uniformly enlarged as much as the control group, although the number of organisms recoverable by culture indicated a lower degree of infection.

Candicidin given at the rate of 0.75 ml daily for 10 days to mice infected with 0.5 ml of 1:100 suspension of *Sporotrichum schenckii* in 5% gastric mucin exerted a pronounced suppressive effect on the disease. The peritoneal surfaces of the intra-abdominal viscera of untreated mice were covered with whitish plaques. Of the 24 treated mice, 6 showed no lesions at all, and the remaining mice exhibited for the most part only a few scattered plaques. This significant suppressive effect should be viewed in relation to the relatively weak *in vitro* effect of candicidin on this organism.

Candicidin administered according to the above dosage scheme was ineffective in protecting 24 mice injected intraperitoneally with a 1:100 suspension of *C. immitis* in 5% gastric mucin.

Discussion. Candicidin is still an impure substance. The different lots available to us varied in antifungal potency and in toxicity. The results must, therefore, be considered as provisional.

The preparations of Candicidin used in these experiments afforded marked protection in moniliasis, blastomycosis, and sporotrichosis of mice. There was a significant but perhaps not strikingly protective effect in histoplasmosis torulosis under the conditions of the experiment. The course of experimental coccidioidomycosis was not affected by this antibiotic.

Candicidin appears to be a promising therapeutic agent because it possesses both fungicidal and fungistatic properties. Little is known about the excretion or destruction of this substance in the body. In its present state of purification, candicidin is too toxic to be injected subcutaneously or intramuscularly. Preliminary studies have shown that it is not absorbed orally. In humans the only possible route of administration would be intravenous infusion of dilute solutions by slow drip.

A potential use of Candicidin would be the prevention of development of a yeast-like fungal flora in the gastrointestinal tract of those receiving such antibiotics as aureomycin, terramycin, and chloramphenicol. Large numbers of organisms belonging to the genus *Candida* become established in the lower gastrointestinal tract of antibiotic-treated humans. In addition, an abundant *Candida* flora arises in the oropharynx of individuals receiving the 3 antibiotics mentioned(3). Certain untoward side reactions are said to be due to this change in the microbial population of the bowel and oropharynx(4). The simultaneous administration of Candicidin with the orally administered antibacterial antibiotics may possibly suppress the development of a *Candida* flora. Our preliminary studies in mice indicate that this is possible. When mice are allowed to imbibe chloramphenicol in their drinking water at the rate of 125 mg/kg for 5 consecutive days, there is a great increase in the yeast flora of the feces. This effect can be entirely prevented by adding 10 mg of Candicidin to the drinking water per day.

Another potential and somewhat unique use for this substance is suggested by the workers at the Communicable Disease Center in Georgia, who have found actidione useful in preventing fungus contamination when trying to isolate organisms such as *C. immitis* and the dermatophytes (the ringworm organisms) (2-5). Saprophytic contaminants, such as *Aspergillus* and *Penicillium,* often interfere with the successful isolation of ringworm fungi. Suppression of these organisms by actidione greatly facilitates isolation. Candicidin would probably have a similar effect.

Summary. The water-soluble fraction of Candicidin has been shown to have strong anti-fungal activity *in vitro* against the major fungus pathogens of man with the exception of *C. immitis* and the ringworm fungi. Candicidin was found to be fungicidal as well as fungistatic. This antibiotic protected mice infected with *C. albicans, B. dermatitidis,* and *S. schenckii.* Candicidin had only a partial

protective effect on torulosis and histoplasmosis of mice.

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Short Persistence of Trichomonas Vaginalis in Reinfected Immune Mice. (20129)

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The chronic tissue infection of mice with *Trichomonas vaginalis*(1) produced by intramuscular injection of the parasites into the hindleg was found(2) to render the animals immune to a reinfection into the leg muscles of the opposite body side. A marked degree of immunity was observed in 70-100% of the animals for periods up to 10 weeks during which the primary lesion persisted. No anatomical lesions and no parasites were found at the site of reinfection in protected animals examined 12 days after the reinoculation. Experiments of this type seemed suitable also to study the fate of the parasites during the early phases of the reinfection. Technically this was facilitated by the strictly localized character of the infection of mice with *T. vaginalis* and because the fate of the parasites could be followed both by microscopical examination and by culture. The results obtained in a group of reinfection experiments are given in this report.

Method. The technic used in the present experiments corresponded closely to that described earlier(2): Adult white mice, from one colony, were infected intramuscularly in the left hindleg with 500,000 parasites contained in 0.5 ml of an overnight culture of *T. vaginalis* in CPLM medium(3). Three to 4 weeks later when, according to earlier experience, a high degree of immunity was present, the immunized mice were reinfected with the same dose of *T. vaginalis* into the muscles of the right hindleg. A few drops of sterile India Ink were added to the culture dilution in order to mark the site of the infection in the tissues. Groups of normal mice received the same infection. At different intervals after the infection, 4 hours, 8 hours, 1 day, 3, 6, 7, 8, and 10 days, groups of 5 to 10 immune and normal mice were sacrificed and the site of the infection was examined microscopically for the presence or absence of motile trichomonads. Cultures in CPLM medium were taken at the same time from the tissues of both the immune and normal groups and examined after 48 hours incubation. The primary lesions of the immunized mice were also examined.

Experimental. A series of 5 experiments, all giving comparable results, was carried out. The data, which are given in Table I, are based on 2 experiments for the intervals of 4 hours and 1 day and on 3-4 experiments for the remaining intervals of 8 hours, 3, 6-8, and 10 days.

All mice of the immune groups were carriers of large abscesses at the site of the primary immunizing infection. The pus of these abscesses contained numerous active parasites which grew abundantly in culture. The fate of the parasites of the reinfection is indicated by the outcome of the cultures taken at the different intervals after the reinfection. In groups no. 2 to 6, covering the intervals from 8 hours to 10 days, the majority of the immune mice did not contain viable parasites at the site of the reinfection. This is demonstrated by the fact that 70-100% of the cul-

TABLE I. Fate of Reinfection with *T. vaginalis* in Muscles of Immune and Normal Mice.
Immunization: 500000 parasites intramusc. left hindleg
Reinfection: 500000 parasites intramusc. right hindleg
Interval between 1st and 2nd infection: 21–29 days
Autopsy: 4 hr to 10 days after reinfection

Group No.*	Interval, days†	No. of mice	Parasites of reinfection			
			Motility	No. of cultures		% negative cultures
				Negative	Positive	
1 I	.17	20	0	4	16	20
1 N	.17	20	0	0	20	0
2 I	.33	30	0	22	8	73.0
2 N	.33	30	0	9	21	30.0
3 I	1.0	20	0	20	0	100.0
3 N	1.0	20	±‡	0	20	0
4 I	3.0	25	0	21	4	84.0
4 N	3.0	30	+	2	28	6.7
5 I	6–8	33	0	27	6	82.0
5 N	6–8	35	+	2	33	5.7
6 I	10.0	20	0	20	0	100.0
6 N	10.0	25	+	0	25	0

* I = immune group; N = normal group.
† Between reinfection and autopsy.
‡ ± signifies that 50% of the normal mice contained motile parasites.

tures failed to show growth of the parasites while in the corresponding groups of normal control animals trichomonads were recovered by culture in the majority of cases. The differences between immune and normal groups were in all these instances significant with 'p' values of 0.01 and less. In the group 1 I, however, sacrificed and examined 4 hours after the reinfection, viable trichomonads were demonstrated in 16 out of 20 mice and the difference between the immune group and the normal group was not significant.

The observation of motility of *T. vaginalis* in direct smears from the infected tissues had essentially the same result, but this method had the disadvantage that marked differences of the immune and normal groups could only be noted after 24 hours and later because motile parasites were also not detected in the smears of the normal controls during the first 8 hours. Marked anatomical lesions could only be expected in the groups 6 I and 6 N examined 10 days after the reinfection. In accordance with earlier observations parasitized abscesses were found in the control group, but were absent in the group of immune mice.

Discussion. The experiments seemed to indicate that the immunity which accompanies the chronic intramuscular infection of mice with *T. vaginalis* exerted an appreciable effect on the persistence of the parasites at the site of the reinfection. The inoculum survived in the tissues for 4 hours but parasites could not be recovered in the majority of instances after 8 hours. The disappearance of viable parasites from the site of the reinfection was a permanent one; the percentage of negative cultures remained on the high level of 70-100% for the entire period of observation up to 10 days after the reinfection. This frequency of immunity under the conditions of this type of active immunization is in good agreement with our earlier observations(2) which were based on the absence of parasitized abscesses in reinfected immune animals.

The findings confirm the general experience in protozoan immunity in which, according to Culbertson(4), the persistence of parasites in immune animals is considerably shortened. In case of *T. foetus* it was shown by Nelson(5) that in experimentally immunized rabbits the duration of a vaginal reinfection was reduced from 23 days to 2 days. The intramuscular trichomonad infection of mice which may persist in normal animals for 12 weeks and longer (1) seemed to be limited in immune animals to a period between 4 to 8 hours.

Summary. In mice immunized by intramuscular infection with live *T. vaginalis* the parasites of a homologous reinfection were found to survive in the tissues of the host for

4 hours. At later intervals from 8 hours to 10 days after the reinfection, viable parasites could no longer be recovered by culture from the majority of animals.

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Alpha Cell Damage and Blood Sugar Changes in Rabbits after Administration of Cobalt.* (20130)

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In a preliminary report it has been shown by us that in the rabbit a single intravenous injection of cobaltous chloride causes both transitory hyperglycemia and severe selective damage to the pancreatic alpha cells(1). Although the blood sugar returned to the pretreatment level within a few hours after cobalt administration, the alpha cell damage was still present after 48 hours. Despite these persistent anatomical changes, a second dose of cobaltous chloride administered at 48 hours induced a second hyperglycemic episode. These observations suggest two possible mechanisms for the action of cobalt. On the one hand it might be assumed that the alpha cells on being damaged by cobaltous chloride released a factor with hyperglycemic properties. This hypothesis would be in conformity with numerous reports which give evidence for the presence of a hyperglycemic glycogenolytic factor (HGF) in the pancreas, and which suggest that the alpha cells are the site of its production(2-4). The absence of a persistent hypoglycemia after the initial hyperglycemia and the repeated hyperglycemic episodes elicited by additional injections of cobaltous chloride may indicate simply that alpha cells sufficient to maintain blood sugar homeostasis and to cause a hyperglycemic phase on readministration of cobaltous chloride have escaped the damaging effects of the initial injection. On the other hand, the alternate hypothesis has to be considered that the alpha cell damage and the hyperglycemic episodes are independent effects of cobaltous chloride, the latter being of extrapancreatic origin. The experiments to be reported in this paper were undertaken to study the interrelationship between the anatomical and functional changes caused by cobaltous chloride.

Material and methods. Cobaltous chloride as 0.5% solution in saline was administered intravenously to normal and alloxan diabetic rabbits of both sexes weighing from 2000 to 3000 g. Alloxan diabetes was produced by the intravenous injection of 100 mg/kilo of alloxan monohydrate (Eastman). Those animals which maintained blood sugar levels over 300 mg for at least one week were utilized. Blood samples for glucose determination by the Nelson modification of the Folin-Wu Micro Method(5) were withdrawn from the marginal ear vein. Animals were sacrificed by intracardiac air embolization and portions of the pancreas and liver were removed for histological examination, which included formalin fixed paraffin sections of both organs stained with Haematoxylin and Eosin, Bouin fixed pancreas prepared by Gomori's Method (6), and alcohol fixed liver stained by the MacManus technique(7).

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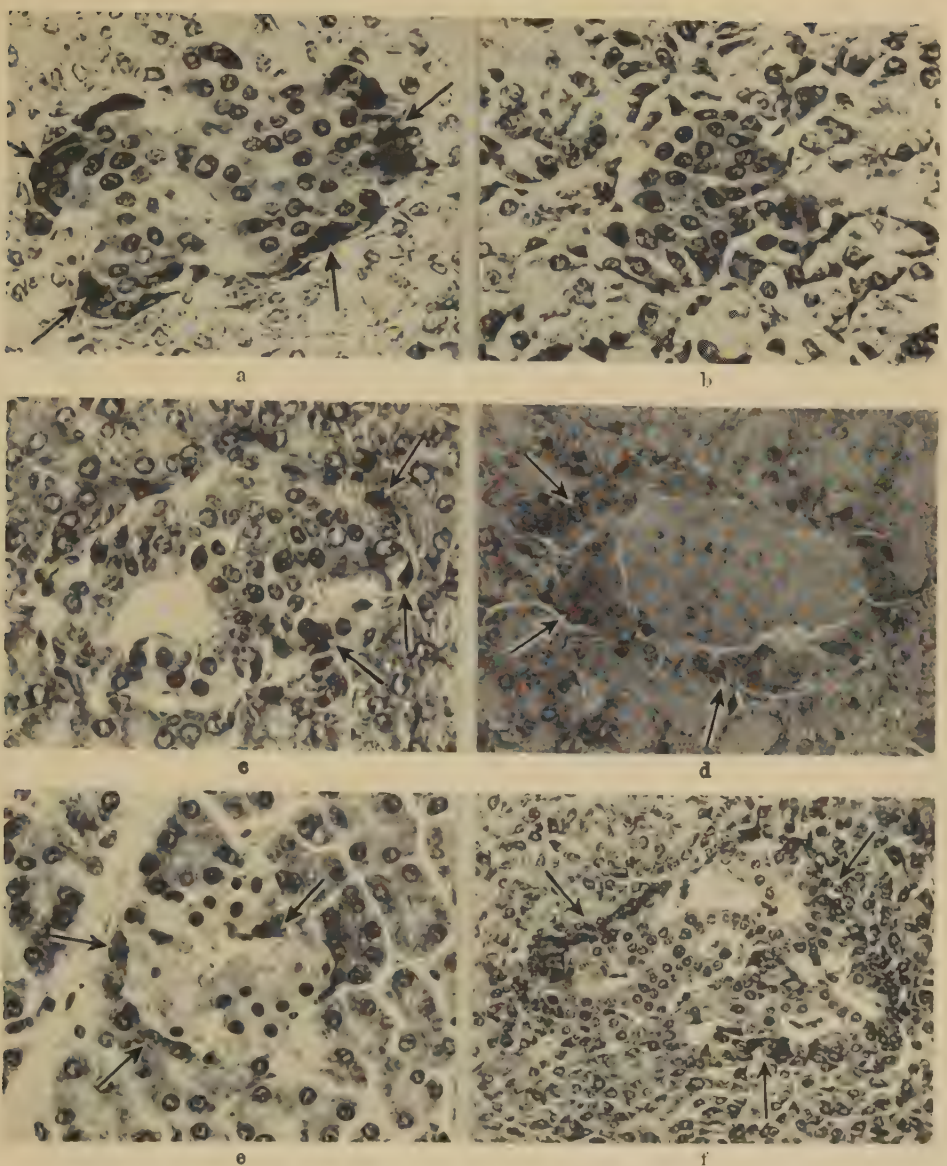

FIG. 1. a. Pancreatic islet of rabbit 1 hr after 50 mg of $CoCl_2$. Arrows indicate degenerating alpha cells. Gomori Stain × 500. b. Same, 3 hr after 50 mg of $CoCl_2$. The islet is composed exclusively of beta cells. Gomori Stain × 500. c. Sections from same pancreas as b, showing a few degenerated alpha cells (arrows). Gomori Stain × 500. d. Pancreatic islet from alloxanized rabbit. Arrows indicate intact alpha cells surrounding debris of the beta cells. Gomori Stain × 500. e. Pancreatic islet from alloxanized cobalt-treated rabbit. Arrows indicate remnants of alpha cells. Gomori Stain × 500. f. Normal control. Gomori Stain × 375.

Results. a. *Optimal Dosage and Toxicity.* An initial series of normal animals each received single intravenous injections of either 20, 40, 50, 80, 100, or 200 mg of cobaltous chloride. The blood sugar fluctuations and morphologic changes were followed as long as the survival of the animals permitted. Those animals receiving 100 mg or more developed wheezing, diarrhea and convulsions and usually died within several hours with symptoms of severe respiratory distress. Animals receiving less than 100 mg of cobalt occasionally

showed diarrhea but no other toxic symptoms. The hyperglycemic response and histologic changes after 20 mg of cobalt were inconstant. Doses of 40 mg to 50 mg seemed to give optimal results. Therefore, a dose of 50 mg was used routinely for the rest of the experiments.

b. *Effect of single and repeated injections of 50 mg of cobaltous chloride.* A single injection of 50 mg of cobalt was given to normal animals and blood samples were withdrawn at half hour intervals for 4 hours, and daily thereafter. Two animals each were sacrificed at 1, 2, and 3 hours, and 1, 2, 4, 6, and 10 days after cobalt administration. Additional groups each received repeated injections of 50 mg of cobalt: 1) at one hourly intervals for 4 doses; 2) at 2 hourly intervals for 3 doses; and 3) at daily intervals for 3, 4, 7, or 10 doses. Animals receiving 3 or more injections of 50 mg each at 1- or 2-hour intervals usually did not survive the third injection. In each case in surviving animals blood sugar determinations were done every half hour during the period immediately following the cobalt injections and at daily intervals thereafter.

Morphologically it was found that one hour after a single injection of 50 mg of cobaltous chloride there was considerable diminution in the number of alpha cells. Most of those remaining were markedly shrunken and rounded in appearance (Fig. 1a). The granules had disappeared and the cytoplasm had become homogeneously acidophilic. The nuclei were compact and pyknotic. Two or three hours after the injection there was a further decrease in the number of alpha cells and many of those still visible were transformed into clumps of acidophilic debris. Many islets at this time consisted only of beta cells (Fig. 1b). In other islets occasional isolated "shadows" of alpha cells were seen at the periphery (Fig. 1c). However, a few intact alpha cells were still visible in most sections. This picture became increasingly accentuated until 48 hours after the cobalt administration. After 4 days these changes were still present. Although after the 6th day the cellular damage was still marked, the number of intact alpha cells was increased, and by the 10th day the regenerative processes were more marked. It should be noted that the effect of cobalt is not entirely uniform in different animals. Since repeated injections of cobalt at hourly and 2-hourly intervals usually caused the death of the animal within 6 hours, serial histologic studies could not be done. However, the most marked alpha cell damage was observed in pancreatic tissue obtained from these animals. Daily administration of cobaltous chloride, on the other hand, did not seem to intensify the anatomical changes produced by a single injection.

Histological examination of the livers failed to reveal any parenchymatous damage but showed marked depletion of glycogen in almost every instance.

The *blood sugar level* after a single intravenous dose of 50 mg of cobalt reached its maximum value between 30 minutes and 2 hours after the injection. The peak blood sugar value averaged 60 mg % above the fasting level. Within 4 hours the blood glucose concentration reverted to the normal range (Fig. 2a). Subsequent daily blood sugar values were not significantly different from those

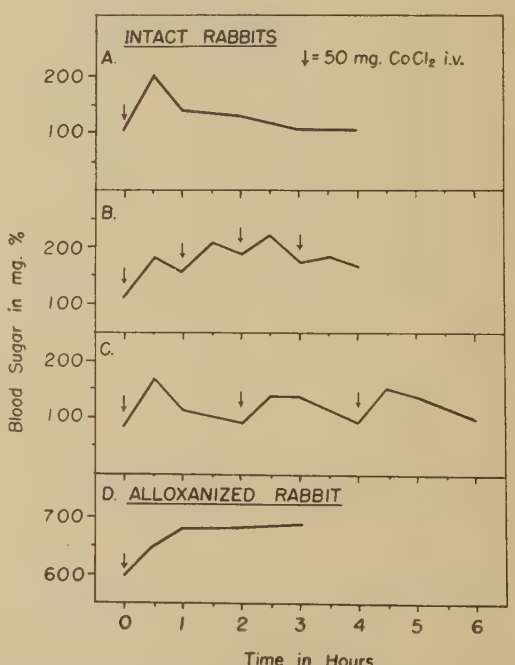

FIG. 2. Initial effect on blood sugar level of single or multiple injections of cobaltous chloride in fasting normal and alloxanized rabbits.

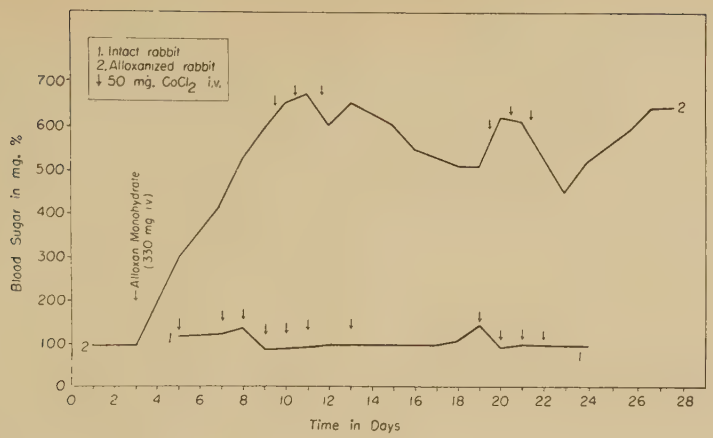

FIG. 3. Effect of daily injections of cobaltous chloride on fasting blood sugar level of normal and alloxanized rabbits.

which would be found in untreated normal animals. Fig. 2b demonstrates the effect on the blood sugar level of consecutive hourly administrations of cobalt, while Fig. 2c represents the effect of injections at two hourly intervals. These repeated injections each cause a rise of the blood sugar level. Repeating the injection of cobalt at daily intervals caused each time a transitory rise of the blood sugar level similar to that seen in Fig. 2a. The interval blood sugar, as seen in Fig. 3, Curve 1, did not vary significantly from the physiologic range.

c. *Effect of cobalt on the alloxanized rabbit.* The alloxan diabetic animals received either single injections of 50 mg cobaltous chloride or repeated injections at daily intervals for 3 days. No insulin was given prior to or during the experiment. Blood samples were taken every half hour for a four-hour period after each cobalt injection and daily thereafter. Animals were sacrificed serially. In several animals the same experiment was repeated at 10-day to 2-week intervals. The islets of control alloxanized rabbits showed the usual morphologic picture consisting of a center composed of granular debris of degenerating beta cells with a peripheral layer of intact alpha cells (Fig. 1d). After cobalt administration this rim of alpha cells showed the same morphologic changes as occurs in the normal islet (Fig. 1e). Most of the alpha cells were replaced by amorphous cytoplasmic remnants. However, it seemed that this destruction of the alpha cells was more marked in the alloxan diabetic than in the normal rabbit. One of our slides showed only undifferentiated cellular debris with an occasional cluster of degenerated alpha cells in the islets.

The effect of cobalt chloride on the blood sugar level of the diabetic animal showed two different phenomena: firstly, each injection was followed immediately by an augmentation of the hyperglycemia which was more prolonged but of the same degree as in the normal rabbit (Fig. 2d); secondly, after the second or third cobalt injection there occurred in some animals a gradual fall of the fasting blood sugar. This continued for several days. After four or five days the blood sugar level, however, returned gradually to the pre-cobalt level (Fig. 3, Curve 2). The alloxanized animal seemed to be more sensitive to the toxic action of cobalt than the normal rabbit and occasionally died after the second or third daily injection with a precipitous fall in the blood sugar.

Discussion. The data presented in this paper favor the hypothesis that the transitory hyperglycemia and the selective damage to the pancreatic alpha cells are independent effects of the intravenous administration of cobaltous chloride.

The morphologic studies demonstrate that the alpha cell damage appears rapidly and is rather extensive. It is observed within one hour after cobalt administration and persists for a period of at least 10 days. Only a few

cells escape serious morphologic damage. Regenerative processes seem to start by the sixth day. The damage induced by a single intravenous dose of 50 mg appears to be almost optimal and can be increased only if the injections are repeated at short intervals of one or 2 hours. Injections at intervals of one or more days do not seem to augment the damage elicited by the first injection.

The changes of the blood sugar, on the other hand, revealed an entirely different pattern. Each intravenous injection was followed by a transitory hyperglycemia, regardless whether the time interval was one or two hours, or 1, 2, or 10 days. The glycemic curve was of the same character and degree after the first injection which caused the most extensive cellular damage as after the subsequent injections which increased this damage only slightly or not at all. Moreover, in the normal rabbit transitory hyperglycemia was followed by a return to normoglycemia within two to four hours in spite of persistent impairment of the alpha cells. This normoglycemia was then maintained indefinitely, or until a new injection caused a new transitory elevation. No periods of hypoglycemia were observed, except in terminal cobalt alloxan diabetic animals, as mentioned above.

If the transitory hyperglycemia were a function of the alpha cell damage, a relationship between its degree and the extent of the cellular damage would be expected; repeated injections of cobalt should cause smaller or no elevations of the blood sugar. The reestablishment and maintenance of normoglycemia in spite of persistent alpha cell damage seems to indicate also that blood sugar homeostasis does not depend on the integrity of these cells unless it is assumed that the few remaining cells suffice for this function.

In the alloxanized animals we found a temporary improvement after cobalt administration, but within a few days the hyperglycemia had returned to the pre-treatment level. It seems possible that a toxic effect on the liver rather than the alpha cell damage caused this temporary lowering of the blood sugar. This is even more likely in view of the death of some of the cobalt treated alloxanized animals with toxic symptoms and a precipitous fall in the blood sugar level. It is well known that hepatic injury ameliorates the diabetes of depancreatized animals(8); reference should also be made to the fact that cobalt is excreted by the organism through the liver and bile(9). It has been stated above that the livers of cobalt treated rabbits appear to be markedly depleted of glycogen.

Our observations are at variance with those of others who noted permanent improvement of alloxan diabetes after the administration of cobalt(10), and who consider the cobalt hyperglycemia as evidence of an alpha cell hormone (11). Further studies are in progress in our laboratory to elucidate the mechanism of the cobalt hyperglycemia in relation to the function of the pancreatic alpha cells as well as on the process of regeneration of these cells after cobalt damage.

Summary. 1. The intravenous injection of cobalt chloride is accompanied by a transient hyperglycemic episode and causes rapid selective injury to the alpha cells of the pancreatic islets which can still be observed after 10 days. However, regeneration of the alpha cells starts by the 6th day. 2. Since repeated cobalt injections each elicit a similar change in the blood sugar level, this is thought not to be due to the alpha cell damage but to an extrapancreatic toxic action of cobalt probably on the liver. 3. Hypoglycemia is not observed in normal rabbits with alpha cell damage, suggesting that the blood sugar homeostasis does not depend on the integrity of these cells. 4. Although a lowering of the blood sugar which is apparently a toxic effect, is observed in alloxan diabetic animals for a few days after repeated cobalt injection, the pre-injection level is regained while alpha cell damage is still present. This is interpreted to signify that the severity of the diabetes of the alloxanized animal is not a result of a physiologic hyperglycemic action of the alpha cells.

The authors wish to acknowledge the technical assistance of Mrs. H. F. Crowley. The photomicrographs were taken by Mr. H. A. Fischler.

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Organelle Nature of a Cell Granule from a Thermophilic Bacterium.* (20131)

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Recent interest in mitochondria of animal and plant cells has quickened the search for specific granules in bacteria(1). In a former paper from this laboratory(2), we presented evidence to show that a fraction isolated from a thermophilic bacterium(3), *Bacillus stearothermophilus* (No. 2184), was, indeed, a specific cell granule. The data rested upon photomicrographs and upon certain enzyme data. Some doubt could still be raised at the organelle nature of the granules on the basis that the particles isolated are not the particles as seen in the intact cells. The percentage of granules in whole cells is roughly 60% of the total protein, a figure which is entirely out of proportion by comparison to figures for organelles of animal cells. Further, staining technics on whole cells could lead to false interpretations due to diffusion artifacts, a fact well recognized by Koelle(4) and Novikoff (5).

Recently, we have obtained data by the use of triphenyltetrazolium chloride (TPTZ) that leaves little doubt as to the existence in the intact cells of the granules obtained after treatment with lysozyme.

Cells of the thermophilic bacterium No. 2184 when treated with TPTZ and substrate reveal zones in the areas where oxidation is carried on due to the deposition of insoluble formazan which can be seen with the phase microscope as highly refractile and remarkably clear areas. Bielig, Kausche, and Haardick(6) have already pointed out the usefulness of TPTZ with the conventional microscope in locating areas of oxidation in bacteria. The granules isolated after lysozyme treatment show the same properties toward TPTZ as do the original cells. In each case where formazan is seen within the cell, the same deposition can be seen in the granule. When no formazan is deposited in the cell, no formazan is deposited in the granules.

The most striking illustration of such parallel behavior is seen with the pyruvic oxidase system. This system oxidizes pyruvate in a solution containing fresh cells or fresh granules (10 mg), diphosphothiamine (50 μg), pyruvate (0.05 M), TPTZ (0.05%), phosphate buffer of pH 6.5 (1.0 ml), magnesium sulfate (4×10^{-2} M) in a total volume of 3.0 ml. At 60°C the reaction time to show pronounced color development is rapid with granules (15 min.) and somewhat slower with whole cells (30 min.). When viewed under the phase microscope after the reaction, the cells show definite refractile areas (Fig. 1). These areas appear to be in the granules themselves, although distinct discernment of the granule is not quite possible without counterstaining, a practice that destroys the for-

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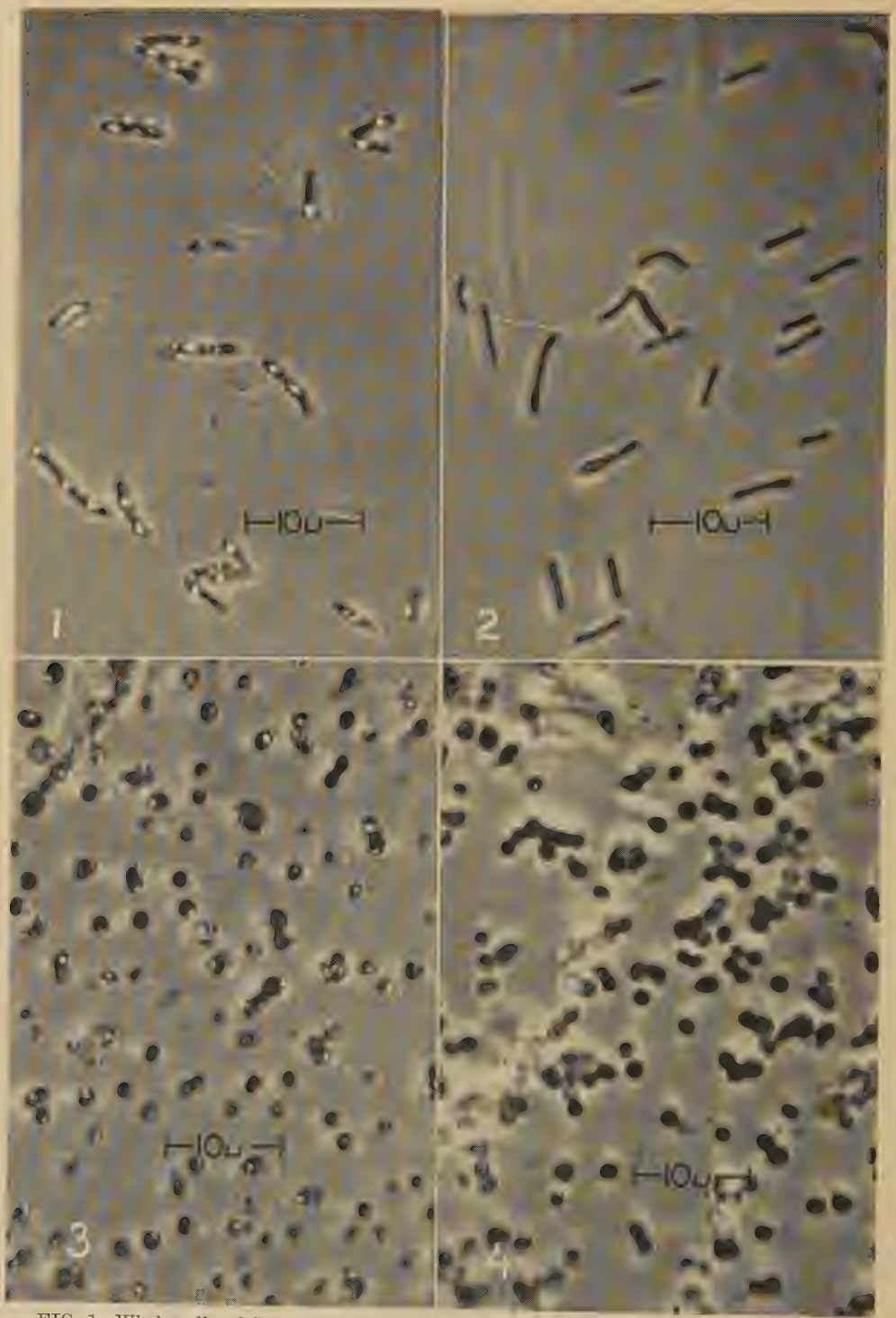

FIG. 1. Whole cells of *Bacillus stearothermophilus* (No. 2184) treated with triphenyltetrazolium chloride (TPTZ) and pyruvate, showing refractile areas under the phase microscope. Cells were immobilized in 1.0% methyl cellulose.

FIG. 2. Control whole cells showing the absence of refractile areas when substrate is omitted.

FIG. 3. Cell granules, isolated from thermophile No. 2184, showing refractile areas with TPTZ and pyruvate.

FIG. 4. Control cell granules.

mazan localization. A control on whole cells without pyruvate does not show refractile areas (Fig. 2) except on prolonged standing in which case the substrates already present within the cells cause deposition of formazan. The isolated cell granules, also, show clear refractile areas with TPTZ and pyruvate (Fig. 3), whereas the control without substrate (Fig. 4) remains negative for days.

The refractile areas in the bacteria cannot be confused as spores which, it could be argued, might arise due to the unusual conditions. A sporulating bacterium can be seen in Fig. 2. The spore, under the phase microscope, does not have the brilliant appearance of the formazan areas.

Cells that have been dried show no activity with pyruvate and TPTZ, nor do the granules isolated therefrom. No refractile areas can be seen under the phase microscope with these preparations. Drying readily inactivates the pyruvic oxidase system, as do freezing and surface inactivation.

Results with other substrates bear out fully those obtained with pyruvate. The granules and the cells show good enzyme activity and refractile bodies with TPTZ and malate, succinate, lactate, glucose, isocitrate, oxalacetate, and fumarate. The supernatant from the separation of the granules shows good enzyme activity only with malate, slight activity at the end of an hour with glucose and lactate, but no activity with pyruvate, succinate, and isocitrate.

In addition to the enzymes mentioned above, the granules show aconitase and alpha ketoglutaric oxidase activity (shown by means other than the TPTZ technic) thus rounding out the group of enzymes making up the Krebs tricarboxylic acid cycle. Table I gives the activity of several of these enzymes. The results with TPTZ on the substrates of the Krebs cycle have been amply verified by studies on oxygen consumption with the Warburg apparatus with the exception of aconitase, which was determined colorimetrically by a method given by Dickman and Cloutier (7).

TABLE I. Activity at 45°C of Some Enzymes Located in the Cell Particle.

Enzyme	Activity/10 mg protein/hr (O_2)
Succinoxidase	36 μl
Cytochrome b	50 "
Alpha ketoglutaric oxidase	47 "
Pyruvic oxidase	254 "
Aconitase	176 μM citrate formed
Isocitric dehydrogenase	59 μl
Malic dehydrogenase	175 "

Conclusions. Photomicrographs and enzyme data obtained with triphenyltetrazolium chloride and various substrates support earlier conclusions that a fraction isolated from a thermophilic bacterium consists of cell granules. Formazan, deposited at local sites of oxidation, appears under the phase microscope as brilliant refractile particles. The isocitric acid cycle appears in the cell granule.

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Phagocytosis Influenced by Bacterial Culture Medium. (20132)

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During a study of opsonins for *Escherichia coli* in the blood of normal humans and dogs, it was noted that the medium in which the organism was grown influenced its phagocytosis. The possibility that a sensitizing material was present in certain types of culture media, or was elaborated during growth in these media, was investigated.

Methods. A strain of *E. coli* used in this laboratory was carried routinely on Bacto Tryptose Agar enriched as noted below. A comparison was made between phagocytosis of organisms grown in Bacto Tryptose Broth to which were added 10 g dextrose, 10 mg $FeSO_4$, and 0.1 mg thiamine hydrochloride per l, and those grown in Bacto Beef Infusion Broth containing 20 g peptone and 5 g NaCl per l. Cultures were grown 18-20 hours at 37°C, harvested by centrifugation, washed in 0.9% NaCl, and resuspended in saline. All live suspensions were standardized to 1.0 x 10^9/ml turbidimetrically, using a Coleman Model 11 spectrophotometer, and were checked by the dilution plate count method. When killed suspensions were employed, 18-20 hour tryptose broth cultures were centrifuged, suspended in saline, and treated with 1% formaldehyde for 24 hours at room temperature. The formaldehyde was removed by 3 washes of saline, and suspensions were checked for sterility by plating. Ultrafiltration of media was carried out with cellophane dialysis tubing, at 5°C and 10 cm Hg pressure. Dog blood was drawn from the jugular vein into a syringe containing 1 mg heparin (Lederle) per 10 ml blood. The cells were washed in 6 changes of Krebs-gelatin solution(1), and then suspended in an equal volume of Krebs-gelatin solution for the phagocytosis test. In the quantitative estimation of phagocytosis, 0.1 ml washed dog blood cells in Krebs-gelatin solution prepared as described above was added to 0.1 ml saline containing 1 x 10^8 *E. coli*. Details of the measurement of phagocytosis are described elsewhere(1).

Results. 1. *Phagocytosis of E. coli grown in tryptose and in beef media. E. coli* were grown 18-20 hours at 37°C, either in tryptose or in beef infusion broth, harvested, and suspended to 1.0 x 10^9/ml in saline. Phagocytosis was measured after rotation of the blood cell-bacterial mixture for 30 minutes at 37°C. The results are summarized in Table I. The mean percentages and their probable errors of neutrophiles phagocytosing *E. coli* after growth in beef and tryptose media were 87.5 $\pm$ 0.8 and 19.4 $\pm$ 1.1, respectively.

2. *Phagocytosis of E. coli grown in ultrafiltration fractions of beef.* In order to obtain information concerning the nature of the principle in beef responsible for increased phagocytosis, sterile beef infusion broth was filtered under pressure through cellophane dialysis tubing. Filtrates varying from 10% to 90% of total original volume were collected, and resterilized by autoclaving. Phagocytosis was equally elevated after *E. coli* was grown in beef infusion medium, its ultrafiltrate, or its residue, Table I, indicating that a responsible principle was freely dialysable and stable to autoclaving.

3. *Sensitization to phagocytosis of E. coli during growth in beef infusion medium.* Since greater phagocytosis of *E. coli* occurred after

TABLE I. Percentage of Neutrophiles Phagocytosing *E. coli* Grown in Beef Infusion Broth, Ultrafiltrate, Residue, and Tryptose Broth.

Medium	No. obs.	Mean $\pm$ P.E.	S.D.	Diff. / P.E. diff.
A. Beef infusion	32	87.5 $\pm$ 0.8	$\pm$ 6.9	
B. Ultrafiltrate	8	92.8 $\pm$ 2.9	$\pm$11.4	A–B 1.77
C. Residue	8	89.8 $\pm$ 3.4	$\pm$13.7	A–C 0.21
D. Tryptose	43	19.4 $\pm$ 1.1	$\pm$11.3	A–D 48.6

TABLE II. Correlation of Growth in Beef Infusion Broth with Phagocytosis of *E. coli*.

Hr of culture	Mean No. of *E. coli*	% of neutrophiles phagocytosing *E. coli*			Comparison of % of 16 hr	
		Mean ± P.E.	S.D.	Diff. / P.E. diff.	Growth	Phagocytosis
Inoculum	5.58×10^8	25.2 ± 4.4	±19.9			30
0	4.79×10^8	28.8 ± 4.6	±20.7	0–I 0.56	8	35
2	3.92×10^9	45.8 ± 4.8	±21.5	2–I 3.17	63	55
4	5.08×10^9	53.2 ± 4.3	±19.4	4–I 4.52	82	63
6	5.94×10^9	72.4 ± 3.8	±17.2	6–I 8.14	96	87
16	6.23×10^9	84.0 ± 2.2	±10.1	16–I 12.00	100	100

TABLE III. Percentage Neutrophiles Phagocytosing Tryptose Grown *E. coli* Washed in Beef Infusion Broth, in 0.9% NaCl, and in Supernatant of Beef Infusion Cultures.

Washing medium	No. obs.	Mean ± P.E.	S.D.	Diff. / P.E. diff.
A. Beef infusion	41	32.7 ± 2.0	±19.4	
B. Saline	39	27.0 ± 1.6	±15.1	A–B 2.19
C. Beef infusion supernatant	8	38.8 ± 3.6	±14.5	
D. Saline	8	33.8 ± 4.4	±17.9	C–D 0.88

growth in beef infusion than in tryptose broth, experiments were designed to determine when sensitization to phagocytosis took place. To 10 ml beef infusion broth was added 1 ml of a 16-hour tryptose broth culture. The *E. coli* were harvested immediately after mixing, and after 2, 4, 6, and 16 hours growth at 37°C. Total number of organisms was estimated turbidimetrically and by dilution plate counts. The suspensions were standardized to 1 x 10^9/ml for phagocytosis measurements. A summary of 10 experiments is shown in Table II. There was a slight loss of organisms from the measured inoculum at 0 hours. The rate of growth was extremely rapid, an 8- to 9-fold multiplication occurring within 2 hours after inoculation. After 2 hours, growth was slower, and approached maximum in 6 hours. Sensitization to phagocytosis increased somewhat more slowly with time, but correlated closely with growth in beef infusion medium.

4. *Phagocytosis of E. coli grown in tryptose and washed in beef infusion broth.* Since growth in beef media resulted in sensitization of *E. coli,* attempts were made to opsonize tryptose-grown organisms by brief contact with beef infusion. When 1 x 10^9 live or dead *E. coli* were suspended in 1 ml beef infusion broth, the liquid removed, and the organisms resuspended in saline, no increase in phagocytosis over saline-treated controls resulted (Table III).

5. *Phagocytosis of tryptose-grown E. coli washed in supernatant of beef infusion cultures. E. coli* were not opsonized by washing in beef infusion medium but were sensitized to phagocytosis during growth in this medium. It was then determined whether the sensitizing factor(s) thus formed diffused into the medium. *E. coli* were grown in beef infusion and in tryptose broths for 18 hours, and removed by centrifugation. The tryptose-grown organisms were suspended in the supernatant of the beef infusion cultures. Volumes of supernatant medium and numbers of organisms were kept the same. After suspending in beef culture supernatant, the tryptose-grown *E. coli* were centrifuged, washed in saline, and again standardized to 1 x 10^9/ml. Controls were tryptose-grown *E. coli* treated with saline. The same experiments with supernatants of cultures were carried out using formaldehyde-killed *E. coli.* Results are shown in Table III. No evidence of free sensitizing factor was found in the beef medium supernatant.

Discussion. Microscopic examination of live organisms or of the stained smears used in the quantitative estimation of phagocytosis has shown that *E. coli* cells from beef medium

are small and rounded, and cells from tryptose medium are larger, more oblongate, and vacuolated. Electron micrograph studies have confirmed these observations. Fenn(2) found greater phagocytosis of larger than of smaller quartz particles, presumably because the probability of collision of particle and of cell depended on their size. The difference between phagocytosis of *E. coli* cells grown in tryptose and beef infusion media cannot be ascribed to size, since the smaller beef infusion grown organisms are more readily phagocytosed.

The foregoing experiments demonstrate that *E. coli* can be sensitized for phagocytosis by growth in beef infusion medium, and indicate that *in vitro* phagocytosis of microorganisms can be influenced by the medium in which they are cultivated. The sensitizing material cannot be washed free; 10- to 12-fold washing in saline did not decrease the phagocytosis of beef-grown *E. coli*. The absence of a sensitizing system from either the original beef infusion or from culture supernatants, and its appearance during growth, indicate its elaboration by the bacteria.

Summary. 1. The medium in which *E. coli* is grown influences its phagocytosis. Organisms grown in beef infusion are phagocytosed much more than those grown in tryptose medium. 2. The factor(s) in beef infusion is ultrafilterable and stable to autoclaving. 3. The absence of opsonin from beef infusion or from supernatant of beef infusion cultures, and the appearance of sensitization during growth, indicate formation of sensitizing substances by *E. coli* in beef infusion medium.

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Comparison of Effect of Progesterone and 11-Ketoprogesterone upon Glycosuria of Partially Depancreatized Rat. (20133)

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The 11-oxygenated steroid compounds of the adrenal cortex are more potent than their 11-desoxy analogs(1) in affecting carbohydrate metabolism. The data of the present study show that 11-ketoprogesterone has a greater diabetogenic effect in the partially depancreatized rat than does progesterone. The first demonstration that 11-ketoprogesterone is diabetogenic was in unpublished experiments by Dr. R. O. Stafford and Mr. W. P. Baker, Department of Endocrinology, The Upjohn Research Division.

Methods. Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they were partially depancreatized at a weight of 275 to 280 g. After diabetes was established all of the rats were placed in metabolism cages and force-fed a medium carbohydrate diet(2) by stomach tube each morning (8:30 to 9:30 a.m.) and afternoon (4:15 to 5:00 p.m.). The technics and diet were modifications of those described by Reinecke, Ball and Samuels(3). The test substances were ground into a fine suspension in peanut oil and were administered by subcutaneous injection twice daily. The animals were kept at a temperature of 74 to 78°F. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 a.m.) and preserved with toluene. Urine glucose was determined by the method of Shaffer and Williams(4). The 11-ketoprogesterone was obtained from the Department of Chemistry of the Upjohn Company, where it was prepared from 11α-hydroxyprogesterone obtained by the microbiological oxidation procedure recently developed in these laboratories(5).

Experiments and results. Exp. 1 (Fig. 1) involved 12 moderately diabetic rats which were observed during a control period of 14 days. Six rats were each treated with progesterone in doses of 1, 2, 4, 8, 16, 32, and 64

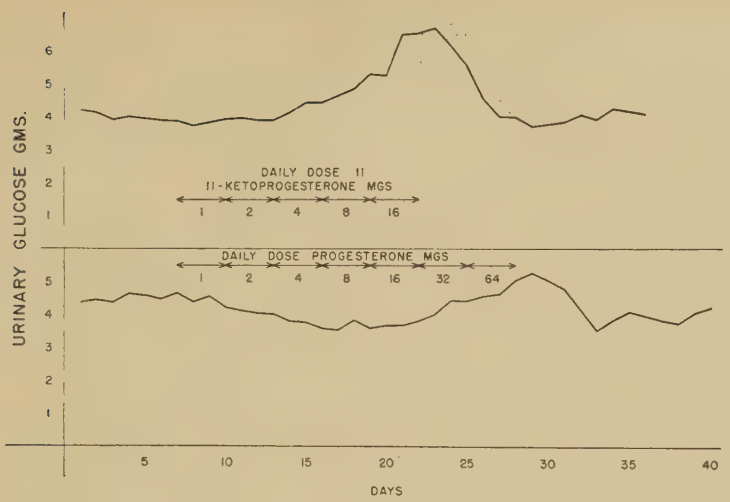

FIG. 1. A comparison of the effects of progesterone and 11-ketoprogesterone upon glycosuria. Averages for 6 rats/group.

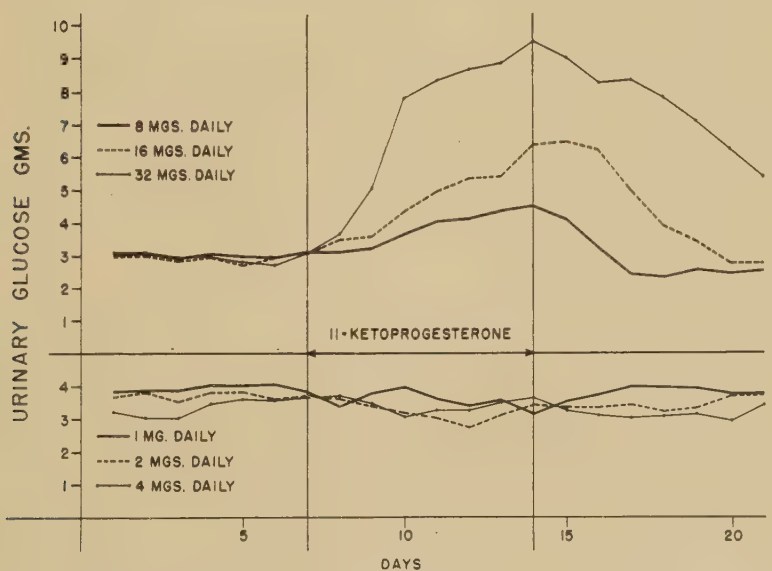

FIG. 2. The effect of different doses of 11-ketoprogesterone upon glycosuria. Averages for 6 rats/group.

mg daily for 3 days per dose. Six rats were each treated with 11-ketoprogesterone in doses of 1, 2, 4, 8, and 16 mg daily for 3 days per dose. When the injections were stopped all of the rats were observed during a final control period of 14 days. During the administration of progesterone in doses of 2 to 16 mg daily there was a small decrease in the average level of glycosuria. At a dose of 64 mg per day there was some rise in the average level of glycosuria. During the administration of 11-ketoprogesterone in doses of 1 to 4 mg daily there was no significant change in the level of glycosuria, but the injection of 8 to 16 mg daily caused a marked increase of the glycosuria of each rat. Equal doses of progesterone were ineffective.

Exp. 2 (Fig. 2) involved 36 moderately diabetic rats which were observed during a control period of 14 days, injected with 11-

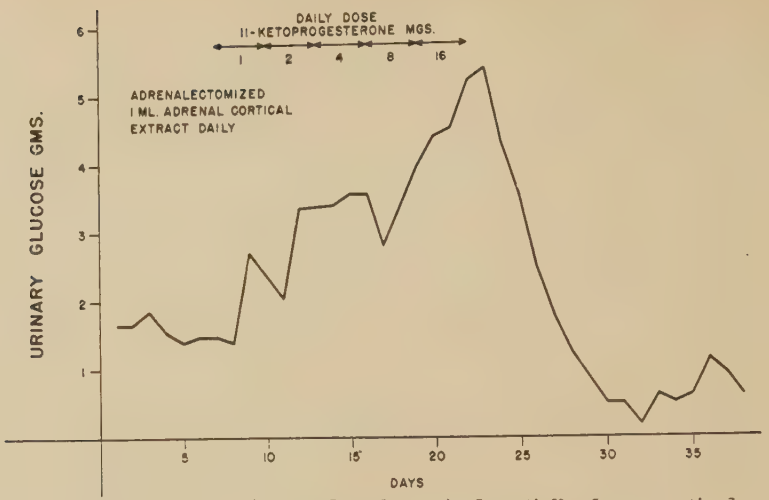

FIG. 3. Exacerbation of diabetes in an adrenalectomized-partially depancreatized rat given 1 ml daily of adrenal cortical extract during the experiment.

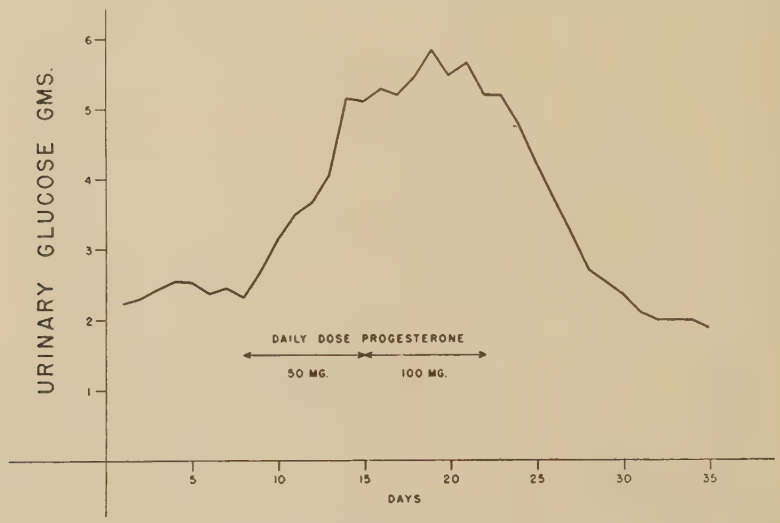

FIG. 4. Exacerbation of diabetes by large doses of progesterone. Average for 6 rats.

ketoprogesterone for 7 days and observed during a final control period of 7 days. Doses of 1, 2, 4, 8, 16, and 32 mg daily were tested on 6 rats per dose. Doses of 1, 2, and 4 mg daily were either ineffective or caused a slight suppression of glycosuria in some of the rats. Doses of 8, 16, and 32 mg daily caused exacerbation of diabetes to an extent which was related to the size of dose. When the injections were stopped the glycosuria fell to pre-injection levels.

Exp. 3 involved 3 adrenalectomized-partially depancreatized rats, each of which was maintained on 1 ml (1 glycogen unit) of adrenal cortical extract per day. Following a control period of 14 days each rat was injected with 11-ketoprogesterone in doses of 1, 2, 4, 8, and 16 mg daily for 3 days per dose. At the end of the injection period the rats were observed during a final control period of 15 days. The injection of 11-ketoprogesterone caused a rise in the level of urinary glucose to an extent that was proportional to the dose. The results are illustrated in Fig. 3.

Exp. 4 (Fig. 4) involved 6 mildly diabetic rats which were observed during a control

period of 14 days, injected with progesterone in daily doses of 50 mg for 7 days and 100 mg for 7 days, and observed during a final control period of 14 days. These doses of progesterone caused significant exacerbation of the glycosuria.

Discussion. Very large doses of progesterone are diabetogenic in the partially depancreatized, force-fed rat. The diabetogenicity of this hormone is enhanced by 11-oxygenation. The diabetogenic action of 11-ketoprogesterone is evident in the absence of the adrenal glands, thereby invalidating the hypothesis that it must be converted to an adrenal cortical hormone by the adrenal cortices before it is biologically active.

Progesterone and 11-ketoprogesterone are each capable of causing partial atrophy of the adrenal glands. The hypothesis was considered that it should be possible to cause some degree of adrenal cortical insufficiency by the administration of these compounds. Since there is a striking amelioration of diabetes in the adrenally insufficient rat we looked for signs of suppression of glycosuria in these experiments. There was a small decrease in the level of glycosuria in some of the rats given 2 to 16 mg daily of progesterone. This small change may or may not have represented suppression of adrenal cortical function. There was no significant suppression of glycosuria by any dose of 11-ketoprogesterone.

Summary. Partially depancreatized force-fed rats were injected with 1 to 100 mg per day of progesterone and 1 to 32 mg per day of 11-ketoprogesterone. Very large doses of progesterone caused exacerbation of the diabetes and 11-ketoprogesterone was found to be significantly more potent in this respect. The diabetogenicity of 11-ketoprogesterone is manifest in either the presence or absence of the adrenal glands.

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Hematologic Studies on Dogs Receiving Low Doses of Total Body Irradiation.* (20134)

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Most studies on the effect of either total or fractional body irradiation on the hematopoietic system have been performed with the application of relatively large doses. The use of such doses produced a multiplicity of changes which probably obscured some of the more basic effects. Therefore, the alteration in the level of circulating granulocytes was not considered to be a satisfactory indicator of the cellular activity in the bone marrow. Accordingly, a detailed study of both bone marrow and peripheral blood cell patterns was made to ascertain the effect of low doses of total body irradiation in both situations.

Method and material. As experimental animals, one- to 2-year-old thoroughbred cocker spaniels and beagle dogs, male and female, weighing from 7 to 11.3 kg were used. These animals were subjected to a total body irradiation of 100 roentgen, given either in single or divided doses. The allocation of the animals to the different groups is shown in Table I. Three cc of cephalic venous blood was drawn into an oxalated 5 cc syringe and transferred at once to a clean test tube. Red and white counting pipets were immediately loaded and counts made in a Neubauer counting chamber. Kingsley stained peripheral blood smears were

* This work was done under contract with the U. S. Atomic Energy Commission.

TABLE I. Allocation of Animals to Experimental Groups.

No. and type dog	r/dose (group)	Doses	Factors kv.	Factors ma.	Filtration	HVL, mm Cu	r/min.
8 cocker	10	10	175	5	None	0.24	12.5
8 "	25	4	"	"	"	"	"
8 "	50	2	"	"	"	"	"
4 "	100	1	"	"	"	"	"
4 beagle	100	1	"	"	.25 mm Cu 1.0 mm Al	0.83	6.7
6 cocker	Non-irradiated control group						

Avg distance from target 66 cm.

TABLE II. Normal Blood Counts for Cockers and Beagles.

Type of dog	No. of Dogs	No. of Counts	Avg Rbc, mill./mm³	Total Wbc, thous./mm³	% neutrophiles (seg.)	% neutrophiles (band)
Beagle	28	36	5.5	13.8	52.0	5.7
Cocker	69	153	5.8	15.6	52.0	7.4

made and most differential counts made by 2 or 3 persons; 200 to 300 cells were counted on each slide. In all irradiation groups blood was drawn 6 hours prior to exposure. Animals in the 10 and 25 r groups were bled 18 hours after each irradiation. Blood samples were obtained 6 and 18 hours after irradiation from the dogs in the 50 and 100 r groups. The control group was bled at the same hour at weekly intervals and was subjected for a period of 3 weeks to similar experimental procedures as that of the other animals but without exposure to x-ray. Blood was drawn 18 hours following such a procedure. From each group, except the control and the 10 r groups, an average of 3 dogs was selected for pre- and post-irradiation bone marrow biopsies taken with a modified Turkel needle(1) from the 6th, 7th or 8th ribs. Both control and treated marrows were obtained from the same animal. Pre-irradiation biopsies were taken approximately one week prior to the initial irradiation. The post-irradiation biopsies were taken 18 hours following completion of 100 r total body irradiation. This biopsy material was fixed with Zenker's acetic acid solution, cut into sections of 5 mμ thickness and stained according to the Kingsley method.

Results. The normal levels for red blood cells, lymphocytes, segmented and band neutrophiles were first determined in both cocker spaniels and beagles (Table II). The effect of irradiation on the peripheral blood pattern is given in Fig. 1-4. The counts in the control groups are given in Fig. 1. Line A represents the total white count; Line B the total neutrophile count; Line C the lymphocytes, and Line D represents the band neutrophiles. This graph shows clearly the small variations occurring during different weeks.

The behavior of the total white, neutrophile or lymphocyte count (Fig. 2) after irradiation with 100 r is similar to what has been previously described. The 50 r group also presented a leukopenic pattern very much like that seen in mice subjected to this dose(2). The 50 r dose appears to be the smallest dose which resulted in this form of sensitivity. The most significant findings were in the 10 and 25 r group. The total and differential counts taken at weekly intervals have been plotted (Fig. 3). The solid lines in this graph connect the average of counts taken 6 hours prior to irradiation while the interrupted lines connect average of counts taken 18 hours following irradiation which was given at weekly intervals for 10 consecutive weeks. Line A shows the total white count which shows only slight variation. The total neutrophile count is indicated by Line B, which shows an elevation during this period of irradiation. The lymphocytic count (Line C) remains fairly constant. The principal change (Line D) is the marked increase of the band neutrophiles in the peripheral blood. This shift to the left is more clearly demonstrated in Fig. 4, in which the band neutrophiles are plotted in the control group, in the 10 r group and the 25 r

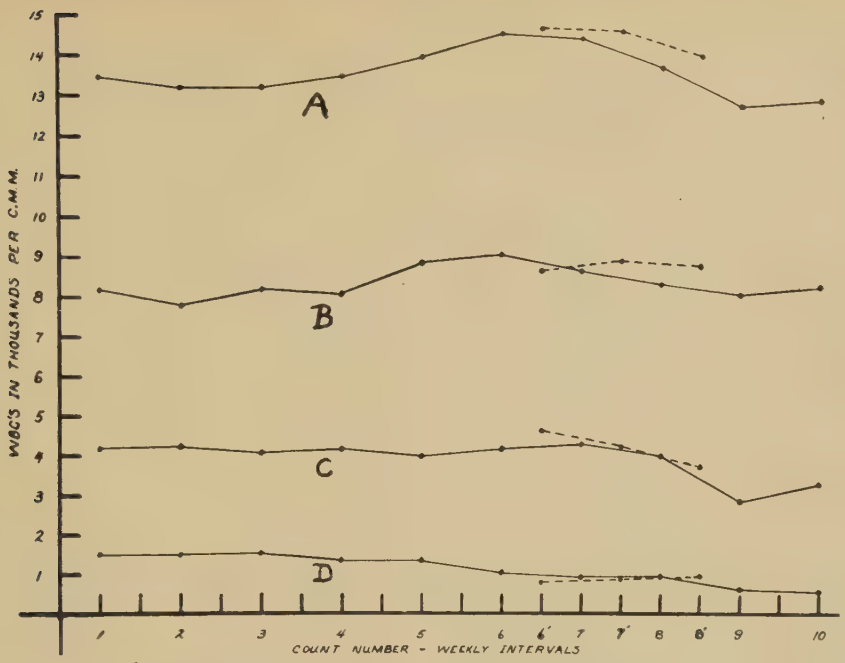

FIG. 1. Blood counts, control group. A. Total white counts. B. Total neutrophile counts. C. Total lymphocyte counts. D. Band neutrophile counts.

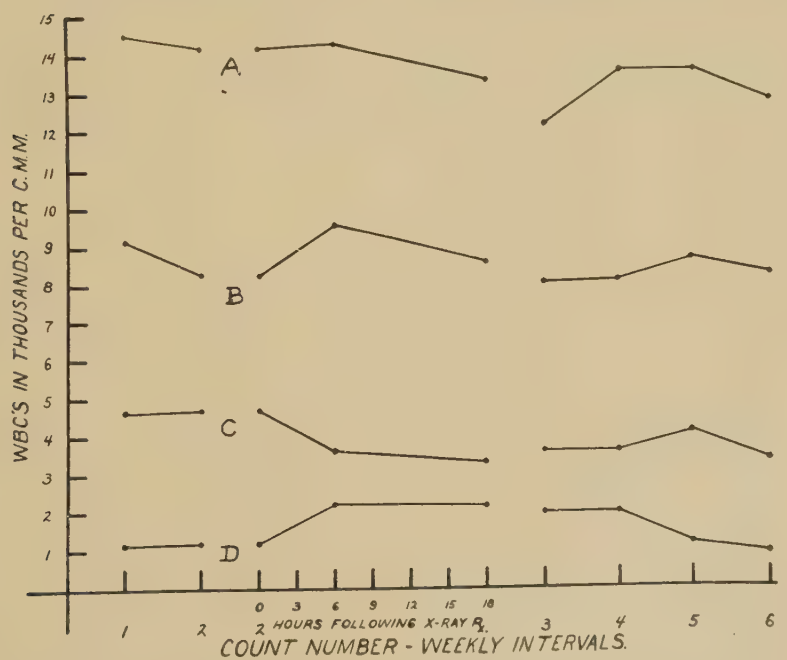

FIG. 2. Blood counts, 100 R group. A. Total white counts. B. Total neutrophile counts. C. Total lymphocyte counts. D. Band neutrophile counts.

group. This graph shows a definite increase of band forms during the irradiation period reaching its maximum in about 8 weeks and followed by a rapid drop to normal. This same increase of band neutrophiles was observed in the bone marrow. A comparison

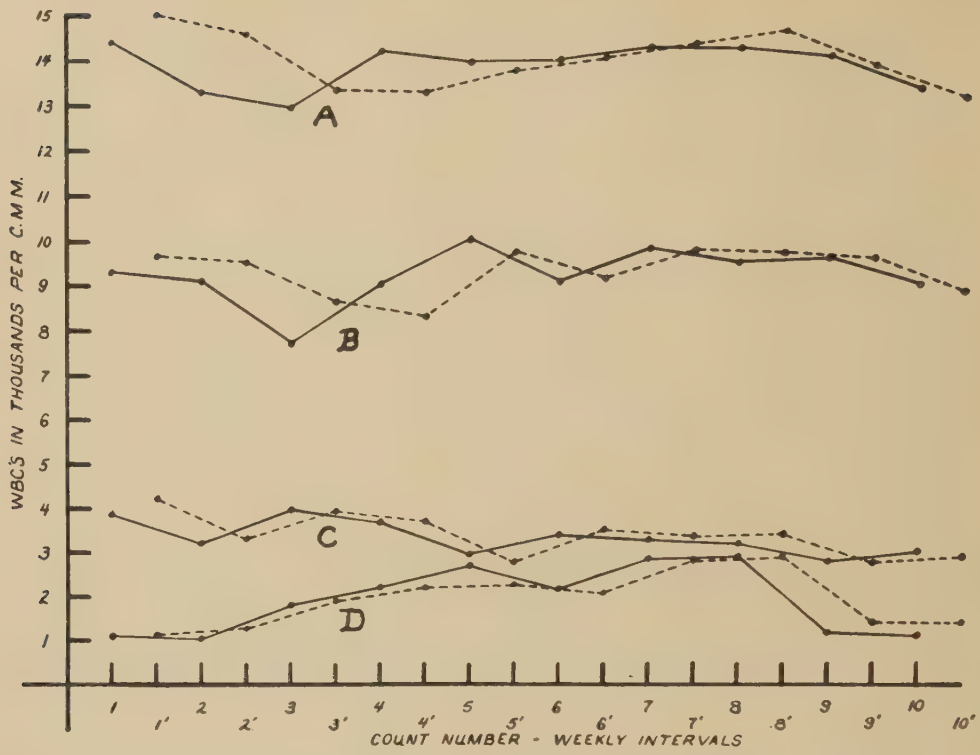

FIG. 3. Blood counts, 25 R group. A. Total white counts. B. Total neutrophile counts. C. Total lymphocyte counts. D. Band neutrophile counts.

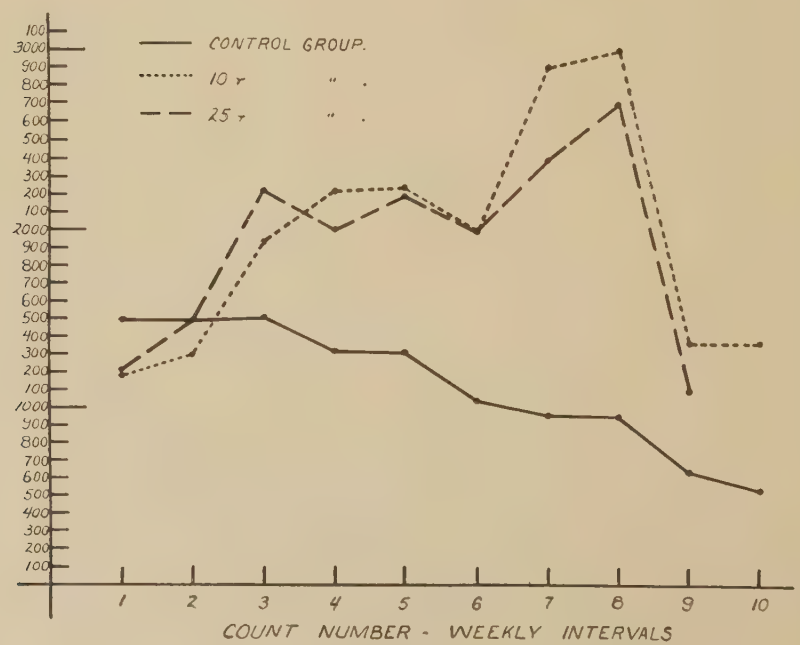

FIG. 4. Band neutrophile count.

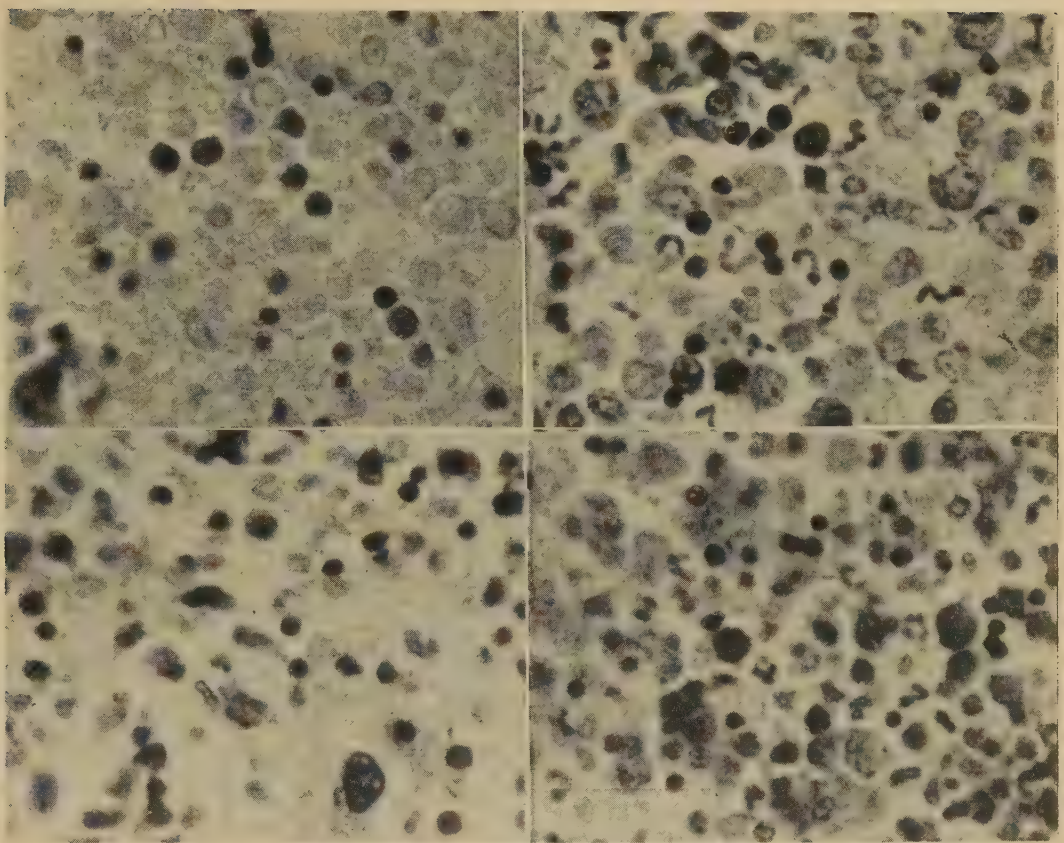

FIG. 5. Bone marrow sections. A, B (left top and bottom). Before irradiation. C, D (right top and bottom). 18 hr after irradiation.

between the pre- and post-irradiation marrows demonstrated an increase in the more mature granulocytes, band as well as segmented forms (3). Bone marrow specimens (Fig. 5) taken from 2 of the dogs before irradiation are compared with specimens taken 18 hours after completion of a total body irradiation of 100 r. The increase in band and segmented granulocytes in the marrow is very pronounced. This change was observed in all 6 animals on which bone marrow specimens were taken before and after irradiation. This increase in the bone marrow occurred at the same time that these cells were markedly increased in the peripheral blood.

Total leukocyte counts taken on the control group 18 hours following simulated irradiation showed a maximum deviation of 200 cells when compared with the counts taken 6 hours prior to this procedure. The total leukocyte counts for both groups during this interval ranged from 13,800 to 14,700 per cu mm. This is within the limit of errors inherent in the method of leukocyte counting(4). It is unlikely, therefore, that the manipulation of the animals in this experiment influenced in any way the data found after exposure to x-ray.

Neither single nor divided administration of this dose in total body irradiation produced any significant changes in the erythrocytes. The results of these counts are summarized in Table III.

Discussion. Our experiments were designed to demonstrate changes in the hematopoietic system at low doses, both single and divided, of total body irradiation. In reviewing the literature, special attention was given to descriptions of early increase in band neutrophiles in the peripheral blood following irradiation in relation to bone marrow activity(5). We felt that such a response could best be studied by parallel samplings of the bone

TABLE III. Red Cell Counts before and after Irradiation.

Period	No. of dogs	No. of counts	Avg Rbc count in mill./mm³
Pre-irradiation	38	75	5.79
Irradiation interval	32	157	5.87
Post-irradiation	21	84	5.83

marrow and peripheral blood. Our results showed that there was a qualitative and quantitative variation in response to 100 r total body irradiation when given in single or divided doses. When 100 r was given in a single dose or in 2 doses of 50 r, the order of decreasing radiosensitivity of leukocytes in the circulation was lymphocytic to granulocytic. However, when 100 r total body irradiation was given in divided doses of 10 to 25 r, this order was reversed. This increase of immature granulocytes in the peripheral blood occurred without evidence of any accompanying leukopenia. The comparison of pre- and post-irradiation bone marrow biopsies showed an increase in band cell granulocytes after exposure in every instance. It is evident, as might be expected, that an increased maturation in the marrow occurs at the time of the elevation of immature band forms of granulocytes in the peripheral blood. Circulating blood may be valuable as an indicator of bone marrow activity following irradiation.

In spite of different opinions in previous articles, we believe that the study of blood and bone marrow samples taken concurrently is an indispensable technic in studying results of irradiation. Experiments in which low dosages of total body irradiation are used offer possibilities for studying reactions of various components of the hematopoietic system which cannot be observed when large doses are employed.

Summary and conclusions. 1. Data showing qualitative and quantitative differences in the peripheral blood and bone marrow of the dog following administration of single or divided doses of 100 r total body irradiation are presented. 2. The accepted pattern of leukocytic radiosensitivity (lymphocytic > granulocytic) is observed after administration of 100 r in one dose or in 2 doses of 50 r. 3. Administration of 10 or 25 r per dose shows a reversal of this sensitivity apparent in the occurrence of an increased number of granulocytes in the blood and bone marrow in the absence of a lymphocytopenia. 4. The increase in band forms in the post-irradiation biopsy specimen indicates an increase in maturation in the bone marrow. 5. The study of the peripheral blood may be valuable as an indicator of bone marrow activity when only small doses of x-ray are used.

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Cholic Acid: Adequate Stimulus for Hyperlipemia in Normal Fasting Rat.* (20135)

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Recent communications from this laboratory(1,2) have reported that experimental increase in the plasma content of cholic acid of the rat caused by either biliary obstruction or by intravenous injection of sodium cholate into normal fasting rats quickly leads to a hypercholesteremic state. Since cholesterol is only one of several lipids known to be increased in concentration in the plasma after obstruction of the bile duct(3,4), it was of interest to determine whether, and to what extent, various plasma lipid fractions of the normal intact rat changed after continuous intravenous injection of sodium cholate.

I. *Methods.* A. *Physiological.* Normal, male, Long-Evans rats were employed in this study. Both the 17 experimental and 9 control rats were operated upon under ether anesthesia and the right kidney removed. The unilateral nephrectomy was done in order to retard the renal excretion of the injected cholate. Then one end of a flexible polyethylene cannula (diameter: 0.011 inch) was inserted into the inferior vena cava of each animal via a lumbar tributary. The other end of the cannula was attached to a previously described apparatus(2) which allowed the continuous intravenous injection of 0.3 ml of fluid per hour. The abdominal incision then was closed and the rats were placed in individual Bollman cages(5). In this manner, the continuous intravenous injection of sodium cholate in Tyrode's solution (equivalent to 75 mg of cholic acid per 1 cc) was given to the experimental rats for 24 hours at a rate equivalent to 23 mg of cholic acid per hour. The control rats received an equal volume of Tyrode's solution. The rats were starved for 12 hours before operation and also during the experiment. Blood samples taken before, and 24 hours after, the beginning of the injection were analyzed for cholesterol, total lipid, phospholipid, neutral fat and cholic acid.

B. *Chemical.* Cholesterol in plasma was determined according to the method of Saifer and Kammerer(6) modified as reported previously(7). Phospholipid was determined according to the method of Fiske and SubbaRow (8) using the modifications of Stewart and Hendry(9). Plasma lipid phosphorus was converted to phospholipid by multiplying by the factor 25. Total plasma lipid was determined as described by Bragdon(10), and neutral fat was determined by difference. Plasma cholic acid (bile acids) was determined by absorption spectrophotometry, as reported in the modification by Wilken(11) of the bile acid method of Minibeck(12).

II. *Results.* The data are presented in Table I. It can be seen that, in the experimental group, each of the plasma lipids approximately doubled in concentration during the 24 hours in which sodium cholate was administered. In this sense, the hypercholesteremic effect of cholic acid is not a specific one, but produces a general and comparable increase in all plasma lipids.

III. *Discussion.* The data show that, under the influence of cholate, the animals become not only hypercholesteremic but also hyperlipemic without any significant change in the ratio of cholesterol to phospholipid in the plasma. In normal humans(4) the ratio of cholesterol to lipid phosphorus is constant despite normal variations in the concentrations of lipid fractions. At pathological levels of cholesterol, this ratio increases rapidly. It seems probable, therefore, that the cholate administered did not derange the lipid metabolism of the rat to such an extent that normal lipid interrelationships could no longer be maintained.

The parallel increase in each lipid class is consistent with a change in the binding properties of the plasma proteins for lipids, brought about by the injection of cholate. The result-

* Aided by grants from the Monterey Heart Assn., the American Heart Assn., and the U. S. Public Health Service.

TABLE I. Cholate Induced Lipemia.

	No. of rats	Avg wt, g	Total lipid, mg/100 cc	Total cholesterol, mg/100 cc	Phospholipid, mg/100 cc	Neut. fat, mg/100 cc	Cholate, mg/100 cc
			Before inj. of cholate				
A. Rats to be given cholate							
	17	247	175	49	47	79	2.1
Range:		211–302	100–276	37–60	13–90	14–164	1.8–2.2
S.E. mean			16	2	7	16	0.03
B. Control rats							
	9	239	145	45	—	—	—
Range:		213–263	114–228	33–65			
S.E. mean			12	4			
			24 hr after inj. of cholate				
A. Rats given cholate							
	17	247	353	106	108	142	19
Range:		211–302	214–650	78–151	34–286	25–285	7–35
S.E. mean			28	5	20	27	3
B. Control rats							
	9	239	154	62	—	—	—
Range:		213–263	124–210	54–72			
S.E. mean			9	3			

ing lipemia is not specific for one type of lipid, as might be the case if a specific chemical bond were established, but is indiscriminate, suggesting a nonspecific form of lipid adsorption. Such an alteration of the lipoproteins of blood so that they are capable of carrying and retaining more lipids has been offered as a hypothesis to explain the hypercholesteremic effect of both Triton WR 1339 (13), and of cholic acid(14).

IV. *Summary.* Experimental hyperlipemia was produced by intravenous injection of sodium cholate into normal fasting rats. The lipemia resulted from a comparable percentage increase in the concentration of each plasma lipid fraction, and not from preferential increase of any one particular plasma lipid.

The authors would like to acknowledge the very valuable assistance of Barbara Gunning, James Ferguson, Eichi Shibata, and Malcolm Smith.

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Effect of Cortisone on Mumps Antibody Formation in Rabbits. (20136)

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It is well established from the work of Germuth(1) and Bjorneboe(2) that, in the rabbit, the administration of cortisone is capable of suppressing the production of antibody to crystalline protein and bacterial antigens. It is the purpose of this paper to extend these observations to the effect of cortisone on the development of antibody in the rabbit to a viral antigen. In order to relate such studies to previous experiments on the cortisone effect, rabbits were simultaneously immunized with mumps vaccine* and with crystallized bovine serum albumin (BSA).† Antibody to BSA was measured by the quantitative precipitin technic and antibody to mumps virus by 3 different serologic tests. The data form, then, a comparative study of the antibody response to 2 different types of immunological stimulus, as measured by several different means, in normal and cortisone treated rabbits.

Materials and methods. Immunization and cortisone schedules. Over a period of 2 weeks, 12 male albino rabbits weighing approximately 2 kg were given three 1 ml intramuscular injections of mumps vaccine, spaced 7 days apart. During this same period these rabbits were also given 7 intraperitoneal injections of alum-precipitated BSA for a total of 10 mg BSA. Beginning 3 days prior to the immunization period and continuing through and for 6 days afterwards, 6 of the 12 animals were daily given 4 mg of cortisone (Cortone, Merck) intramuscularly. The cortisone treated animals remained in apparent good health and showed no appreciable weight loss. All rabbits were bled from the ear prior to and at 7, 14, 21, 42, and 46 days after the immunization period. *Complement-fixation test.* Lederle mumps vaccine was used as antigen. In the test 2 units of antigen were used, usually necessitating a 1:8 dilution of the vaccine. The test was carried out in a final volume of 1 ml made up of 0.2 ml each of antigen, complement and serum and 0.4 ml of sensitized sheep cells. The end point was taken as the serum dilution producing 3+ or greater fixation of complement after incubation at 37°C for one hour and is expressed as the initial serum dilution. *Antihemagglutination test.* The procedure used was that reported by Robbins, Kilham, Levens, and Enders(3), except that incubation was for one hour at 37°C rather than overnight at 4°C. All sera used in the test were pretreated with cholera vibrio filtrate prepared as follows: a tryptose phosphate broth culture of the *Inaba* strain of *V. comma* was incubated at 37°C for 16 hours. The broth was then passed through a Seitz filter and, if sterile, stored at 4°C until used. In the test, serum and filtrate were mixed 1:5, incubated at 37°C overnight, then incubated at 56°C and used immediately. Dilutions are expressed as the final serum dilution. Treatment with the cholera filtrate efficiently removed the nonspecific mumps antihemagglutinin of rabbit serum without affecting the specific antibody titer. *Virus neutralization.* The Enders strain of mumps virus was cultivated in embryonated eggs and the allantoic fluid harvested, pooled and titered for egg infectivity. Neutralization tests were made with aliquots of this pooled fluid. The sera were inactivated at 56°C for 30 minutes and serial 2-fold dilutions of serum incubated with approximately 1000 egg infectious doses of virus for one hour at 37°C. Each dilution was then inoculated into 8 or more embryonated eggs by the allantoic route. Egg infection was then determined by the presence of hemagglutinin and the egg protective dose (EPD_{50}) was calculated by the method of Reed and Muench(4). *BSA antibody N.* A quantitative precipitin curve was obtained on each serum by adding increasing quantities of BSA to 1 ml portions of the antiserum, and collecting the precipitates after 48 hours in the cold. The precipitates were

* Lederle.

† Armour.

TABLE I. BSA and Mumps Antibody in Control Rabbits.

Rabbit No.			Pre-imm. bleeding	Bleeding–days after last dose of antigens 7	14	21	42
7	BSA Ab	*	—	165	248	155	—
	Mumps Ab	HI	<20	320	160	80	80
		CF	ac	16	16	< 4	<4
		Ne	< 5	20	18	<10	—
8	BSA Ab	*	—	91	121	76	—
	Mumps Ab	HI	<20	320	160	80	80
		CF	ac	16	16	< 4	<4
		Ne	< 5	12	13	<10	—
9	BSA Ab	*	—	202	218	147	—
	Mumps Ab	HI	<20	640	640	320	160
		CF	< 4	32	16	4	<4
		Ne	< 5	38	24	14	—
10	BSA Ab	*	—	111	276	153	—
	Mumps Ab	HI	<20	640	640	320	320
		CF	< 4	32	16	8	4
		Ne	< 5	40	37	11	—
11	BSA Ab	*	—	226	397	304	—
	Mumps Ab	HI	<20	640	320	160	160
		CF	< 4	32	16	4	<4
		Ne	< 5	32	21	<10	—
12	BSA Ab	*	—	204	265	193	—
	Mumps Ab	HI	<20	640	320	160	160
		CF	< 4	32	16	4	<4
		Ne	< 5	30	13	<10	—

* $\frac{\mu g \text{ Ab N}}{\text{ml antiserum}}$

HI = antihemagglutinin titer; CF = complement fixation titer; Ne = neutralization titer; ac = anticomplementary.

washed twice with cold saline and the N then determined by a micro-Kjeldahl procedure. The supernates were analyzed for antigen and antibody.

Results. The cortisone-treated animals had much lower levels of circulating anti-BSA than did the controls (Tables I and II). At the 7-day interval the cortisone rabbits had only about 25% of the antibody in the controls. In further agreement with the work of Germuth(1) were the results of an Arthus reaction done on all animals on the 7th post-immunization day, prior to bleeding. Injection of 1 mg of BSA intracutaneously resulted in much larger reactions in the control than in the cortisone treated group.

The mumps antibody shows a somewhat different picture. The control series showed that all the animals responded to the mumps vaccine in a fairly uniform manner, as measured by any one of the 3 serologic tests used. Moreover, there was a good general correlation of results obtained by the 3 methods so that rabbits showing the higher level of mumps antihemagglutinin also showed higher levels of complement-fixing and virus neutralizing antibody. The mumps antibody in the cortisone-treated animals showed levels which, on the average, were lower than those in the control group, but relatively not as low as the BSA antibody. Thus, the averages of mumps antibody measured by hemagglutination-inhibition ranged from 50 to 60% of the averages of the antibody levels in the controls. In considering individual animals, rabbits 3 and 5 had antibody levels very similar to those in the control group, yet the BSA antibody in these same rabbits was markedly suppressed. At 42 days after cessation of cortisone a booster dose of mumps vaccine (0.5 ml) was given and antihemagglutinin tests performed on the sera from all the animals 4 days later. All rabbits showed a rise of antibody to 320 with the exception of rabbit 4, whose response

TABLE II. BSA and Mumps Antibody in Cortisone Treated Rabbits.

Rabbit No.			Pre-imm. bleeding	Bleeding–days after last dose of antigens 7	14	21	42
1	BSA Ab	*	—	14	26	10	—
	Mumps Ab	HI	<20	80	80	40	40
		CF	< 4	4	<4	<4	<4
		Ne	< 5	<5	<5	<5	—
2	BSA Ab	*	—	51	52	32	—
	Mumps Ab	HI	<20	80	80	80	80
		CF	< 4	16	16	8	4
		Ne	< 5	<5	<5	<5	—
3	BSA Ab	*	—	57	77	64	—
	Mumps Ab	HI	<20	640	640	320	80
		CF	< 4	32	32	16	4
		Ne	< 5	17	12	10	—
4	BSA Ab	*	—	68	37	12	—
	Mumps Ab	HI	<20	80	40	40	20
		CF	< 4	4	4	4	4
		Ne	< 5	<5	<5	<5	—
5	BSA Ab	*	—	31	46	22	—
	Mumps Ab	HI	<20	640	320	160	160
		CF	< 4	32	16	8	4
		Ne	< 5	19	17	12	—
6	BSA Ab	*	—	6	9	0	—
	Mumps Ab	HI	<20	160	160	80	80
		CF	< 4	ac	8	4	4
		Ne	< 5	<5	<5	<5	—

* $\frac{\mu g \text{ Ab N}}{\text{ml antiserum}}$.

HI = antihemagglutinin titer; CF = complement fixation titer; Ne = neutralization titer; ac = anticomplementary.

was 80. This indicates that all the animals, when not under the influence of cortisone, were capable of responding to mumps vaccine to about the same degree with the exception noted. Rabbit 4, then, was probably a "poor antibody producer." This animal was included in the averages, but had it been omitted the mumps antibody averages would have been still closer to the control figures.

Discussion. The data obtained in the present experiments confirm the observation that cortisone administered to rabbits suppresses the level of antibody produced to purified protein antigens (BSA) and extends this finding to include antibody to mumps virus vaccine as measured by serologic methods. Under the experimental conditions herein employed, however, it is apparent that the suppression of mumps antibody was not as great as that of BSA antibody. The explanation for this is not clear from the present data. It may be that mumps virus is more antigenic than BSA, but this does not seem likely. Another possible explanation may be the difference in sensitivity of the methods used for detecting antibody. Thus, the serologic methods can only measure variations of 2-fold or greater so that a 40 to 50% lower level of antibody might not be detected serologically but would be readily measured by the precipitin technic. However, we feel that the relative insensitivity of the serologic measurements do not account for the fact that the BSA antibody levels in all the cortisone-treated animals were suppressed to the same marked degree, yet 2 of these animals by 3 different (and repeated) serologic tests showed the same levels of mumps antibody as did the controls. Further studies with antigen-antibody systems to which both quantitative and serologic methods can be applied may clarify this point and such experiments are now in progress.

Summary. 1. Serial antibody studies were performed on normal and cortisone treated rabbits immunized with mumps virus and bovine serum albumin. 2. In rabbits, corti-

sone suppresses development of antibodies to mumps virus as measured serologically, although the suppression is to a relatively lesser extent than that for BSA antibody measured quantitatively.

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Renal Functions During Positive Pressure Respiration. (20137)

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Although the circulatory changes during respiration at pressures above atmospheric have been extensively studied, the effect of such a procedure on renal functions has not been investigated, except by Drury, Henry, and Goodman(1), who showed that marked reduction in urine volume and urea clearance resulted.

Methods. Eight experiments have been done on dogs in barbiturate anesthesia, in which respiration at pressures from 9 to 31 mm Hg above atmospheric was brought about by connecting the trachea with a large chamber through which air was passed at a rapid rate. Measurement of intratracheal pressure with a Hamilton manometer showed it to be close to the chamber pressure and essentially constant throughout the respiratory cycle. In 7 of the experiments, 1 kidney was denervated by stripping all visible nerves from artery, vein, and ureter. In 3 experiments, the vagus nerves were cut, to avoid respiratory inhibitory reflexes which limited the height to which the pressure could be raised. This did not appear to alter the type of response. In 5 of the experiments, pressure pulses in the aorta were recorded and cardiac outputs calculated from them(2). In all experiments, priming doses and continuous infusions of creatinine, p-aminohippurate, and glucose were given. Blood plasma and urine were analyzed for these substances, and clearances and maximum tubular transfer calculated. In most experiments, plasma and urine samples were analyzed for sodium and potassium by flame photometry, in some, urine chloride only was determined. Venous pressure was measured in some experiments.

Results. The experiments may be divided into 2 groups, one in which the TMg fell, one in which it rose. For the first group, the values, in the innervated kidneys, at maximal change were, in % of control (average, with range) C_{cr} *62* (33-85), C_{pah} *73* (43-100), TMg *70* (50-94). In the second group, these were C_{cr} *88* (63-115), C_{pah} *91* (76-103), TMg *187* (111-193). As an example of group 1, it can be seen in Fig. 1 that on the application of the pressure, the fall in cardiac output is associated with a fall in arterial pressure, but this returns to normal with an increase in total peripheral resistance. While the kidney appears to take part in this reflex vasoconstriction, with parallel decreases in clearances and TM, its response does not develop maximally until the third ten-minute period, by which time cardiac output and total peripheral resistance have returned essentially to normal. When respiration is returned to atmospheric pressure, there is a rise in cardiac output and a fall in total peripheral resistance, and renal functions return to normal, but as can be seen in the values for RF, that fraction which renal blood flow is of total blood flow, the kidney again only slowly takes part in the total change. This lag in response of the kidney was seen in the experiments cited above(1), and may resemble that seen in hemorrhage(3).

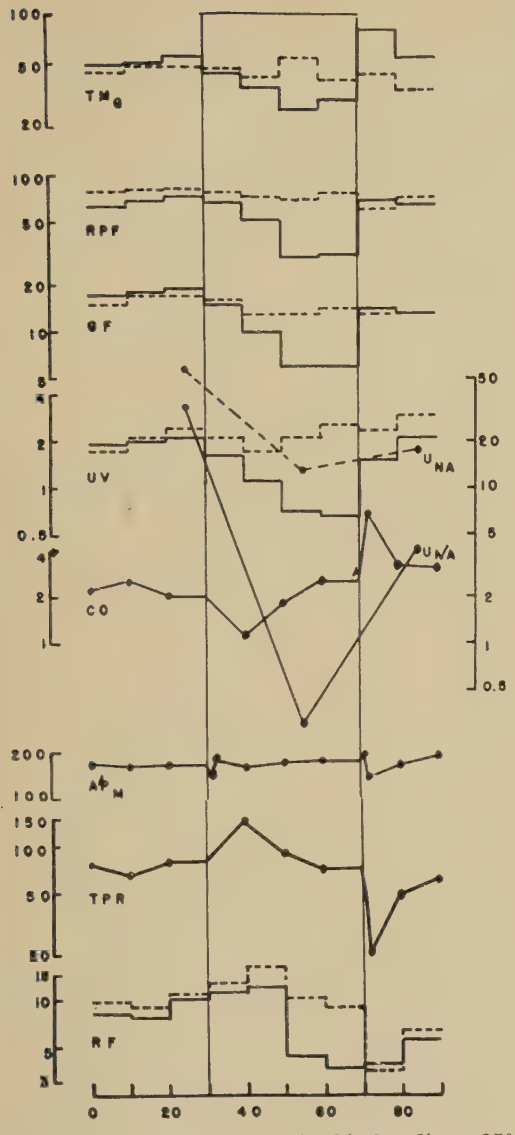

FIG. 1. Male dog, 10.9 kg barbital sodium, 250 mg/kg. TM_g, glucose tubular maximum, mg/min. RPF, renal plasma flow from p-aminohippurate clearance, ml/min. GF, glomerular filtration rate from creatinine clearance, ml/min. UV, urine volume, ml/min. UNa, urine sodium excretion, microequivalents/min. CO, cardiac output, liters/m^2/min. AP_m, mean arterial pressure from planimetry of pulse curve. TPR, total peripheral resistance, AP_m divided by CO, arbitrary units. RF, renal blood flow in % of CO. Solid lines are innervated kidney, dashed lines are denervated kidney. Time in min. Respiration of positive pressure during periods 4-7, tracheal pressure 14 mm Hg.

Drury, *et al.*, took into account a possible humoral mechanism for reducing urine volume, but this can scarcely be the case here. The fact that the denervated kidney does not take part in the phenomena just described indicates that they are nervous rather than humoral in origin.

The marked reduction in sodium excretion, to 1% of the control rate, should be noted. The much smaller change in the denervated kidney may result from the increased venous pressure, as has been shown to occur without change in filtration rate(4). Potassium excretion showed a fall also, but to 5% in the innervated, 60% in the denervated kidney.

In the other type of response, as seen in Table I, the general circulatory changes were similar, but falls in glomerular filtration, renal blood flow, and urine formation were small or absent, and TM values rose. In this experiment, at the end of the last clearance period Janus green was injected into the left ventricle and the kidneys removed ½ minute later, with following estimation of the number of active glomeruli(5). The proportion between the 2 kidneys in glomerular count supports the TM as a measurement of number of nephrons.

It may be that in this type of experimental result, not all nephrons were active in the control state, but tended to become so during pressure respiration, thus preventing a reduction in filtration and blood flow in the kidney as a whole, although these values were reduced in the individual nephron. Since these changes occurred in both kidneys, they seem to be independent of the nerves, and may represent some intrinsic mechanism of control.

Summary. Dogs under barbiturate anesthesia with one kidney acutely denervated and respiring air at greater than atmospheric pressure show two types of renal functional changes. In half the animals studied, a reduction in glomerular filtration rate, renal plasma flow, maximal tubular transport of glucose, urine volume, and sodium excretion occurred in the intact kidney. No change in these functions occurred in the denervated kidney. In the rest of the animals, changes in glomerular filtration, renal plasma flow, and urine formation were smaller, while the TmG increased. The renal functions of these animals appear to have been depressed during control observations, perhaps due to trauma or deep anes-

TABLE I.

Female dog, 19.5 kg pentobarbital Na 30 mg/kg. Right kidney denervated; vagi cut. No. of glomeruli, left 231600, right 177900. UV urine volume, UCl urine chloride, C_{cr} creatinine clearance, C_{pah} p-aminohippurate clearance, TM_g glucose tubular maximum, RR renal resistance, AP_m divided by renal blood flow, AP_m, mean arterial pressure from planimetry of Hg manometer tracing.

UV, ml/min.		UCl, μEq/min.		C_{cr}, ml/min.		C_{pah}, ml/min.		TM_g, mg/min.		RR		AP_m,	
Inn.	Den.	Inn.	Den.	Inn.	Den.	Inn.	Den.	Inn.	Den.	Inn.	Den.	MM Hg	Procedure
2.9	1.6	60	43	27.9	14.2	182	129	48	40	34	48	154	c*
3.1	1.5	50	25	26.9	12.4	204	124	45	35	31	51	156	c
3.5	1.5	59	15	24.9	10.6	198	122	39	32	37	59	171	c
4.0	1.3	67	16	25.8	10.6	188	120	50	39	35	55	158	25 mm Hg†
4.7	1.3	87	12	30.7	12.1	199	116	85	54	31	53	160	31

* c = control. † Pressure resp.

thesia, but positive pressure respiration caused partial restoration. Since both kidneys participated, the mechanism must be independent of the renal nerves.

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Prophylactic Penicillin in Anterior Ocular Transplantation Technic in Mice.*† (20138)

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The animal anterior ocular chamber offers an unusual opportunity to study the behavior of transplanted tissue in that the progress of the graft may be easily observed, and several animals may be used in the same case, with sacrifice at different ages, microscopic studies, and retransfer. A vast field for clinical application of this technic was opened by Greene(1) when he grew human sarcomas and carcinomas in the anterior ocular chambers of foreign species. So as to obviate the ever present risk of extraneous infection, there is need to improve technics in dealing with such implanted tissue. Investigators rarely report one hundred percent incidence of tissue revascularization in anterior chamber transplants. Although it is true that many problems are involved, a major factor preventing successful transfer is infection, arising either from originally contaminated tissue or from contamination of the operative site at the time of transfer. Penetration of penicillin into the eyes of dogs(2) and rabbits(3) has been measured by several investigators. Prophylactic penicillin was used systemically (4) in the anterior chamber transplantation of ovarian tissue in rabbits, however, controls were not used.

The purpose of this investigation is to evaluate the effectiveness of systemic penicillin in lowering the infection incidence in the anterior chamber technic of tissue grafting, utilizing the mouse eye.

Materials and methods. Three hundred and eighty-five, white Swiss, C3H and hybrid

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TABLE I. Lung Tissue Transplants.

Group	Infected	Non-infected
A. Penicillin	66	31
B. Untreated	62	33

TABLE II. Corneo-scleral Incisions.

Group	Infected	Non-infected
C. Penicillin	42	53
D. Untreated	45	53

(C3H X white Swiss) were used. The mice were kept in clean glass jars and fed mice food pellets and water *ad libitum*. The animals were divided into 4 groups: Group a) received anterior chamber grafts with systemic penicillin; group b) received anterior chamber grafts without penicillin; group c) were prepared by making corneo-scleral incisions into the anterior chamber while penicillin was given systemically; and group d) received corneo-scleral incisions without penicillin. Corneo-scleral incisions were made in all animals with a Deutschmann cataract knife. In the group with anterior chamber transplants, fragments of lung tissue from newborn to 6-day-old mice were used as graft material. This tissue was placed into a blunt, beveled, 20-gauge intravenous needle and then injected into the anterior ocular chambers of chloroformed mice, in the manner described by Greene(5). Strict asepsis was maintained in the technic. In those mice receiving antibiotic therapy, penicillin G was given in arbitrary amounts of 5,000 units per day intraperitoneally for 3 days beginning on the day of the transplantation or incision of the eye. All of the eyes were enucleated eight days after the operation, fixed in formalin, embedded in paraffin, cut at 7 μ, stained with hematoxylin and eosin and examined microscopically.

Discussion. The inflammatory reactions within the histological sections of the mouse eyes varied from a limited, chronic, inflammatory response about the incision area to a severe panophthalmitis; however, only the absence of all inflammatory reaction was accepted as evidence of lack of infection. Penicillin was ineffective in lowering the incidence of inflammation, both in the group of mice prepared only with incisions into the anterior chamber and in the mice receiving transplants. Several explanations may be presented for the apparent failure of the penicillin. Primarily, the criteria for "non-infected" were quite rigid. It is conceivable that some degree of inflammatory response would be present even in the absence of infection. It should be stressed that the very size of the graft used in the mouse eye necessitates more manipulation than does the graft used in larger animals. It was assumed that the total therapeutic dose of penicillin was approximately 100 μ/cc of aqueous humor, *i.e.*, 1000 μ/g of recipient mouse). There was, however, a quantitative degree of difference between the treated and untreated groups. Somewhat less severe inflammatory response was present in the eyes of the mice receiving penicillin therapy. This may be an indication that there was some suppression of the infection by the penicillin. The incidence of vascularization of the lung transplants was not evaluated since serial sections of the eyes would be necessary in order to visualize all of the graft tissue. Suffice to say that a number of grafts became revascularized, while others regressed.

Summary. Of 192 mice with anterior chamber implants of newborn mouse lung, 97 were treated with systemic penicillin. An additional 193 mice were prepared with corneo-scleral incisions into the anterior ocular chamber and 95 of this group received systemic penicillin. Beginning on the day of operation, penicillin was given in 5,000 unit doses over a 3-day period. The eyes were enucleated after an interval of 8 days, and studied microscopically. Seventy percent of mice receiving penicillin exhibited inflammation and/or infection in the transplanted eye, while 65% of the untreated mice with transplants had inflammatory and/or an infectious response in the transplanted eye, the difference not being statistically significant. We may conclude that prophylactic penicillin at the therapeutic levels utilized is of no value in reducing the inflammatory response in the experimental anterior chamber technic of lung transplantation in the mouse eye.

The author wishes to acknowledge the valuable assistance of Drs. B. L. Newton, Department of

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Poliomyelitis Virus in Human Blood During the "Minor Illness" and the Asymptomatic Infection.* (20139)

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Viremia in human poliomyelitis has been reported previously from this laboratory, in one child out of 111 tested(1). The positive result was obtained with blood drawn from a 9-year-old girl 6 hours after the onset of symptoms in an abortive attack of poliomyelitis. At the time, these results were considered evidence that viremia is of rare occurrence in the human disease, and probably not of importance in its pathogenesis. Subsequently, it became known that antibodies are already present at the time of onset of CNS signs both in patients(2,3) and in primates orally infected in the laboratory(4,5). Thus our negative results had to be reinterpreted, for it is clear that if viremia occurs, it must do so early, either in the incubation period or *minor illness* phase, and in 110 of our patients we had probably looked for it too late. The possibility of viremia in the experimental disease had been recently restudied in laboratory primates, and 2 reports(6,7) indicate that the presence of virus in the blood is a regular feature after oral and other routes(8) of administration. In cynomolgus monkeys and in chimpanzees, viremia occurs as early as between the 3rd and 8th days after its ingestion or cutaneous injection, and as long as a week *before* the appearance of paralysis. In reinvestigating viremia in human poliomyelitis, therefore, efforts have been concentrated on obtaining specimens from contacts of cases, *i.e.*, children who might be in the incubation period, and from children with the minor illness syndrome in the midst of an epidemic. The present report deals with the findings in one infected family, in which 3 of the 4 children had minor illnesses during a poliomyelitis epidemic in their community.†

Procedure. Family epidemiology. The W. family resides in the small town of Burbank, Ohio, where an epidemic of poliomyelitis occurred during the summer of 1952(9). The family had had no known contact with a frank case of the disease, but beginning June 20, 21, and 22, the 3 older children aged 10, 4, and 3, developed mild, febrile illnesses, while the youngest, aged 2, remained well. Slight fever, anorexia, nausea and vomiting, headache, and sore throat, in various combinations were the complaints. The oldest child complained also of soreness of the calves of the legs. The father, aged 37, had noted a headache on June 19, but this had disappeared and there were no associated symptoms. The mother remained well throughout. All of the illnesses were compatible in their symptomatology

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† These specimens were collected by one of us (R.W.Mc.) in Wayne County, Ohio. Our thanks are due to Dr. E. E. Kleinschmidt, Health Commissioner of the Wayne & Medina General Health District and to Dr. Fred Wentworth, Chief of the Communicable Disease Division, Ohio State Department of Health and their staffs, and to Mr. Edward Tulley, Chairman of the Summit County Chapter of the National Foundation for Infantile Paralysis, for their generous cooperation. We are also indebted to Mr. Claude Reed, Miss Ilah Kaufman, and Mrs. Berttina Orsborne, for assistance in the collection of specimens.

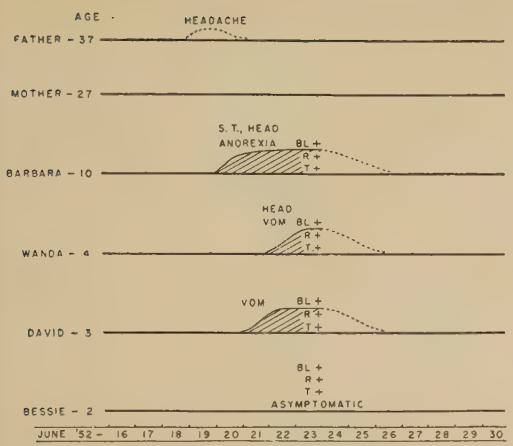

FIG. 1. Diagram of a family outbreak of poliomyelitis in which 3 cases of the minor illness occurred. Shaded areas indicate period of fever. S.T., sore throat; head., headache; vom., vomiting. + refers to results of tests for virus in blood (Bl), rectal swabs (R), and throat swabs (T).

with abortive poliomyelitis(10). They were of short duration, and none was followed by any sequellae. A schematic diagram of the illnesses appears in Fig. 1. *Collection of specimens.* On June 23, at the height of the family epidemic, throat swabs, rectal swabs and blood specimens were collected by one of us (R.W.Mc.) from the 4 children. Throat and rectal swabs were preserved in 50% sterile glycerine, and frozen on dry ice. The bloods were collected in "vacutainer" tubes containing potassium oxalate, and were centrifuged for 10 minutes at 3000 rpm to layer the plasma and red cells before being frozen on dry ice. All materials were transported to New Haven in the frozen state, and kept frozen until tested. *Testing of specimens.* Both tissue culture tubes and rhesus monkeys (*M. mulatta*) were used in testing blood specimens, while tissue culture alone was employed for the testing of throat and rectal swabs. The roller tube method, with monkey testicular tissue was used(11), except in a few tests in which monkey kidney tubes were employed. With the exception of one blood specimen (Bessie, aged 2), which was tested before and after ultracentrifugation at 30,000 rpm for one hour, the plasma and/or whole blood was inoculated directly into a minimum of 4 tubes. If no degeneration occurred, the 8- and 12-day harvests of all 4 tubes were pooled and a 2nd passage using 2-5 tubes was made before the test was considered negative. Several tests were carried out with each blood, returning each time to the original specimen. For monkey inoculation, 0.6 ml of plasma, or plasma and red cells mixed, was inoculated intracerebrally, 0.3 ml being directed into either side of the thalamus. The injection was repeated after 3-4 weeks, if the animal remained well. All monkeys showing any suggestive signs of poliomyelitis were sacrificed for histological examination. *Identification of virus strains.* Tissue culture neutralization tests(11) were carried out on all strains isolated. A series of tubes was set up with equal parts of the unknown virus harvested from a tissue culture passage, and Type 1, Type 2, and Type 3, hyperimmune monkey serum, diluted 1:5. A control tube contained virus and balanced salt solution in equal parts. The mixtures were incubated at room temperature for one hour before being inoculated into 2 tubes each. The test was read at 4 and 8 days, and complete inhibition of fibroblastic degeneration by serum of one type only was taken to indicate specific neutralization of the virus.

Results. Poliomyelitis virus was isolated from the blood of all 4 of the children—from the asymptomatic youngest child, as well as from the 3 older ones who had symptoms. All 4 also had virus in throat and rectal swabs. A summary of the results correlated with the clinical course, is indicated in Fig. 1, and the details of the virus isolations are given in Table I. All strains isolated proved to be Type 1 (Brunhilde).

The amount of virus present in the positive blood specimens must have been minimal for it was insufficient to produce poliomyelitis on direct intracerebral inoculation into rhesus monkeys. On first passage in testicular tissue roller tubes, the results were only suggestive, but on 2nd and 3rd passages, characteristic fibroblastic degeneration of increasing severity occurred. Third tissue culture passage from 2 of the specimens was inoculated into rhesus monkeys, and produced typical poliomyelitis, clinically and histologically. One blood specimen (from Bessie, aged 2) was positive in only one of 3 tests using testicular tubes; after ultracentrifugation it yielded a strongly

TABLE I. The Isolation of Type 1 Poliomyelitis. Virus from blood, throat and rectal swabs of 4 children in one family.

	Age	Date onset	Specimens collected 23/6/52	Tests for poliomyelitis virus: Direct monkey inoculation	Tissue culture passage: 1st	2nd	3rd	Monkey inoc., 3rd T.C. passage
B	10	20/6/52	Blood	0/1*	2/3	4/4	2/2‡	1/1
				0/2†	2/3	4/4		
			Throat swab		2/2	2/2	2/2‡	
			Rectal swab		1/2	2/2	2/2‡	
W	4	22/6	Blood	0/2	0/4	0/2		
					3/5	3/3	2/2‡	1/1
					1/3	1/2		
			Throat swab		2/2	2/2	2/2‡	
			Rectal swab		2/2	2/2	2/2‡	
D	3	21/6	Blood	0/2	0/4	0/2		
					0/4	0/3		
					0/5	1/5	2/2‡	
					3/10K	2/2‡K		
			Throat swab		4/4	2/2‡		
			Rectal swab		2/2	2/2	2/2‡	
Be	2		Blood	0/2	0/4	0/2	0/2	
					0/4	0/4		
					1/6	5/6		
					9/10‡§K	2/2K		
			Throat swab		2/2	2/2	2/2‡	
			Rectal swab		2/2	2/2	2/2‡	

* Denominator, No. inoculated; numerator, No. positive.
† These animals received 0.3 cc intracerebrally once only.
‡ Indicates specific neutralization by Type 1 antiserum.
§ Specimen ultracentrifuged before being tested in kidney tissue tubes.
K indicates kidney tissue roller tubes.

positive result in kidney tissue tubes (Table I).

Discussion. The isolation of poliomyelitis virus from the blood of an asymptomatic child and from 3 children with symptoms of the *minor illness* confirms our earlier observation from this laboratory of viremia in a child with abortive poliomyelitis. As with the earlier case(1) all of the children were excreting virus in the stools at the time it was found in the blood. Evidence is thus accumulating that viremia can occur in the human infection as it does in chimpanzees and cynomolgus monkeys, and, as with the latter, it is *not* associated with the clinical appearance of CNS signs, but occurs several days earlier, in association with the minor illness, or in the asymptomatic infection, probably several days after exposure. Whether or not viremia can occur also in the incubation period of the single or double phase illness has not yet been demonstrated. In any event, the presence of viremia early in the course of *human*—as well as *experimental* infection—supports earlier hypotheses(12-14) of rapid virus multiplication outside the CNS. Where this multiplication occurs, whether in neural or non-neural elements, is not as yet certain. Bodian(15) believes that primary multiplication somewhere in the intestinal mucosa, followed by discharge of virus into the blood stream, which carries it to the CNS, is the most likely sequence of events. Faber *et al.*(16) on the other hand, are of the opinion that the source of circulating virus in orally infected cynomolgus monkeys is early multiplication in peripheral ganglia; they discount the possibility of multiplication or spread in non-neural tissues. The problem can only be resolved by demonstrating directly the early presence or absence of virus in various neural and non-neural tissues after various routes of administration.

Summary. Poliomyelitis virus has been isolated from the blood of 4 children in one family during a poliomyelitis epidemic in Ohio in 1952. Three of the children had characteristic clinical pictures of the *minor illness* or abortive poliomyelitis, and one was asympto-

matic. All were found to have virus in the throat and rectal swabs as well as in the blood. None went on to develop signs or symptoms of the *major illness,* either paralytic or non-paralytic.

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Standards for Hepatic and Hematologic Tests in Monkeys: Observations During Experiments with Hepatitis and Mononucleosis.* (20140)

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(Introduced by C. V. Seastone.)
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The paucity of available data on the values for liver function tests and blood counts in monkeys has suggested that publication of such information might be useful to other laboratories interested in investigating chemical or infectious agents suspected or capable of producing hepatic damage in monkeys.

The observations recorded in the present report were obtained during the course of unsuccessful attempts to transmit viral hepatitis and infectious mononucleosis to monkeys. It had been our hope that administration of adrenocorticotropic hormone (ACTH), cortisone, or urethane might induce susceptibility to these diseases in a laboratory animal otherwise resistant. Under the conditions of these experiments, this did not occur.

Materials and methods. Twenty normal monkeys (16 rhesus and 4 cynomologous) were bled from the femoral vein prior to inoculation and the following tests of liver function carried out by standard methods; total and one minute serum bilirubin, thymol turbidity, cephalin cholesterol flocculation, and alkaline phosphatase. Later in the study bromsulfalein dye retention (5 mg/kg) was determined 45 minutes after inoculation. Leucocyte counts were done in duplicate and differential counts were done by enumeration of 200 cells on Giemsa stained glass slides. Fourteen of these twenty monkeys were then inoculated with materials obtained from the patients in the active stage of characteristic viral hepatitis. Except for one sample of pooled plasma which had produced serum hepatitis in 10 or 20 human recipients(1),‡ it was not known whether SH or IH virus was represented. In addition to the hepatitis materials, 8 of these monkeys received injections of cortisone, 3 received ACTH, and 1 was fed

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TABLE I. Results of Liver Function Tests and Blood Counts in 20 Normal Monkeys.

Name of test	No. of tests	Unit	Result			
			Mean	Range	S.D.	±2 S.D.
Total serum bilirubin	25	mg %	.10	.08– .18	.033	.04– .17
1′ serum bilirubin	25	mg %	.06	.03– .12	.007	.04– .07
Thymol turbidity	25	unit	.93	.25– 2.00	.40	.14– 1.72
Cephalin flocculation	25	48 hr	0	0	0	
Alkaline phosphatase	14	unit	17.46	8.2 –25.1	4.49	8.5 –26.5
Leucocyte count	24	×1000*	13.4	7.7 –20.7	3.36	6.7 –20.1
Lymphocytes	24	%	63.0	31 –85	12.20	38.0 –87.0
Neutrophiles	24	%	35.2	14.0 –67.0	12.50	10.0 –60.0
Eosinophiles	24	%	1.7	0 – 7.0	1.88	0 – 5.46

* No. of cells/mm³.
S.D. = Stand. dev. ±2 S.D. includes 95% of the observations.

urethane in milk. Six other monkeys received materials by various routes obtained from patients early in the course of infectious mononucleosis in conjunction with injections of ACTH. Following inoculation with these various materials, the monkeys were bled weekly or oftener, for liver function studies and blood counts over a period of 75 to 116 days in the hepatitis experiments and for approximately 50 days in the infectious mononucleosis experiments.

Results. Normal values. A statistical analysis§ of the values for liver function tests, leucocyte counts and differential blood counts in 20 normal monkeys prior to inoculation is shown in Table I. The highest total and one minute serum bilirubin values observed were 0.18 and 0.12 mg % respectively. The thymol turbidity value never exceeded 2.0 units and the cephalin cholesterol flocculation test was consistently negative. In contrast to these results, which are in a much lower range than is considered normal for man, the alkaline phosphatase was comparatively very high with an average value of 17.42 units. Later in the study the bromsulfalein dye excretion test was carried out 35 times in 14 monkeys. The average BSP retention at 45 minutes was 1.58 mg % with a range from 0 to 2.5 mg %. During the course of the experiments described in the next section, 185 additional determinations of serum bilirubin, cephalin-cholesterol flocculation and thymol turbidity were made. Except for 3 slight increases in serum bilirubin and in thymol turbidity all results fell within the normal limits shown in Table I. *Leucocyte counts* in normal monkeys varied from 7700 to 20000 per cubic mm with a mean of 13400 per cubic mm and the differential counts showed similar variation with a range in lymphocyte percentages from 31 to 85. Considerable variation was encountered in blood counts of individual monkeys from day to day.

Experiments with viral hepatitis and infectious mononucleosis. The design and results of experiments attempting the transmission of viral hepatitis to monkeys are summarized in Table II. In most instances cortisone, ACTH, or urethane was administered in conjunction with materials from patients with viral hepatitis in the hope of altering host-virus relationships. The results of these studies were essentially negative; during a period of 75 to 141 days no monkey became clinically ill, showed significant fever, or had more than slight and transient changes in liver function tests.‖ Thus, as shown in Table II, monkeys RH 3 and RH 28 both had increases in total serum bilirubin (TSB) to 0.22 mg %, one on the 10th day after inoculation of pooled hepatitis sera, and the other 72 days after the initiation of a 5-day period of inoculation with liver suspension from a fatal case of hepatitis. Both of these monkeys also received cortisone. Monkey 886 had rises in TSB to 0.28 and 0.32 mg % on the 25th and 53rd day respectively after the initiation of a series of repeated injections of liver suspension from 2 fatal cases of hepatitis. None of these 3 monkeys with slight increases in TSB had alterations in other liver function tests. Three other monkeys had

§ Appreciation is expressed to Major W. A. Haendiges, 98th General Hospital, for the statistical analysis.

‖ Monkey 889 died on 205th day after inoculation of uncertain cause.

TABLE II. Summary of Attempts to Produce Viral Hepatitis in Monkeys.

Monkey No.	Hepatitis inoculum		Other inoculum				Length of observations (days) †	Result
	Type	Route and method	Type	Inoc. schedule*	Daily dose (mg)	Total dose (mg)		
CY 001 RH 2	Pooled early sera	Multiple parenteral	Cortisone	—2 to + 5	50	350	75	Rise in TSB in RH2 to 0.22 mg % on 10th day
30	Proved S.H. sera	IV and SQ	"	—2 to +14	25	400	141	Negative
889	Duodenal juice Stool suspension	Parenteral Oral	"	—1 to + 1	25	75	103	"
27	Liver biopsy susp. Stool suspension	Parenteral Oral	"	—1 to + 1	25	75	103	"
26	Liver biopsy susp., then convalescent sera 3 wk later	Parenteral	"	—1 to 0	25	50	117	"
28 29	Liver susp. of fatal cases	IP daily for 5 days	"	—2 to +14	25	400	139	Rise in TSB in RH28 to 0.22 mg % on 72nd day
15 7 CY 004	Pool of early sera. Pool of early duodenal juice, both repeated once	Parenteral Gastric tube	ACTH	—2 to +19	40	840	115	Negative
RH 884	Proved S.H. sera	IV and SQ	Urethane	− 3 to +57	500 (oral)	30500	141	"
886 887	Liver susp. of 2 fatal cases followed in 10 days by pooled acute phase sera	IP daily for 4 days IV	0				116	Rise in TSB to 0.28 and 0.32 mg % in RH 886 on 25th and 53rd day respectively

* Minus and plus refer to days before and after inoculation of hepatitis materials respectively; 0 day is the day of inoculation.

† Period during which liver function tests were done. Monkeys were observed clinically for 6 months.

IV = Intravenous; SQ = Subcutaneous; IP = Intraperitoneal; CY = Cynomologous; RH = Rhesus monkey; SH = Serum hepatitis. It is not known whether the other materials represented SH or IH.

slight and isolated increases in thymol turbidity above 2.0 units, the highest being 3.25 units.

None of the 6 monkeys inoculated with materials from patients with infectious mononucleosis in conjunction with ACTH administration developed clinical, hematological, or serological evidence of this disease and in none were alterations in liver function tests seen that exceeded the normal limits shown in Table I.

Discussion. This paper has reported unsuccessful attempts to transmit viral hepatitis to monkeys. Inocula consisted of sera, liver biopsy material or stool samples obtained from patients early in the course of viral hepatitis or of liver suspensions from fatal cases of this disease. These materials were given either alone or in conjunction with the administration of ACTH, cortisone, or urethane. Liver function tests performed weekly over a period of 75 to 141 days remained within normal limits. It is conceivable but unlikely that the incubation period might be longer than this in monkeys. Similar unsuccessful attempts were made to transmit infectious mononucleosis to monkeys receiving simultaneous injections of ACTH. The ability of adrenal hormones to increase the severity of certain infections in susceptible hosts is now well established as emphasized in a recent review(3), but in accord with the results of this paper it has been difficult to induce susceptibility to an infection with these hormones in a species naturally resistant.

In conjunction with these experiments an analysis was made of the results of liver function tests carried out in 20 normal monkeys prior to inoculation. With the exception of the alkaline phosphatase, all the results fell within a narrow range and the standard deviation was small. The reliability of these results as standards for normal is further supported by the fact that 182 of 185 additional tests of liver function carried out in the course of the experiments described were also within this normal range. It should be emphasized that the mean value, as well as the range of values, for each of these liver tests in monkeys represents very low figures as compared to values for these tests in normal humans. For this reason, human standards must not be used to interpret liver function tests in monkeys. This is particularly true of the total serum bilirubin in which the upper limit of normal in the monkey is only ¼ of that of normal man. Thus an abnormal serum bilirubin, elevated as much as 4 times above normal in a monkey might be overlooked if interpretation were based on human standards.

An exception to these low values was found to be the alkaline phosphatase. Results for this test in normal monkeys were consistently higher than seen in normal man. This may depend on a more actively metabolizing osseous system rather than on liver function *per se*.

Leucocyte counts and differential blood counts in normal monkeys were found to have great variation not only within the species but in individual monkeys. Similar findings have been reported by other investigators as summarized by Wintrobe(2).

Summary. 1. The inoculation of materials from patients with viral hepatitis or infectious mononucleosis into monkeys either alone or in conjunction with adrenocorticotropic hormone, cortisone, or urethane failed to result in evidence of successful transmission of these diseases. 2. Normal values for liver function tests, leucocyte counts, and differential blood counts in monkeys are recorded.

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Effects of Dimenhydrinate and Diphenhydramine on Apomorphine-Induced Emesis in Dogs and Cats.* (20141)

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Despite the wide use of dimenhydrinate (Dramamine) and diphenhydramine (Benadryl) as anti-emetics, almost nothing is known about their mechanism of action. In many experimental studies on the problem, apomorphine has been used as the emetic stimulus. Wang and Borison(1) have found that apomorphine does not act directly on the medullary vomiting center but through the closely situated chemoreceptor trigger zone. This introduces one more possibility for the site of the alleged protective action of Dramamine and Benadryl against drug-induced vomiting. Such a possibility is strengthened by the recent finding of Wang and Chinn(2) that destruction of the chemoreceptor trigger zone in dogs prevents motion-induced vomiting. However, before the intimate mechanism of action of these anti-emetic agents can be elucidated, it is necessary to delineate accurately their specific effects in apomorphine-induced emesis. Chen and Ensor(3) reported a significant reduction in the severity but not in the incidence of apomorphine-induced vomiting in dogs pretreated with Dramamine or Benadryl. Similar results were subsequently published by Ducrot and Decourt(4). On the other hand, White and coworkers(5) reported *complete* protection with these drugs against apomorphine-induced vomiting in dogs. In contrast, Moser(6) failed to find any inhibition of vomiting induced in dogs by either subcutaneous apomorphine or oral copper sulfate. Similarly, Freese and associates(7) have reported no protection by Dramamine against morphine-induced emesis in dogs. (Presumably, the emetic action of morphine is identical with that of apomorphine.) Mitchell(8), in experiments on 3 cats, found that the emesis induced by subcutaneous apomorphine was prevented by Dramamine in 13 out of 15 trials, whereas Benadryl afforded protection in only 2 out of 12 trials.

The series of experiments reported herein was performed on dogs in which individual intravenous emetic threshold doses of apomorphine were carefully determined so that each animal could serve as its own control for subsequent tests with Dramamine‡ and Benadryl.§ In addition we attempted to confirm certain experiments, reported in the literature, which were interpreted as demonstrating that these agents afforded protection against apomorphine-induced emesis.

Methods. Five dogs were tested to determine the minimal, individually effective i.v. dose of apomorphine which consistently evoked emesis. The appropriate dose of apomorphine HCl was given in approximately one ml of a freshly made aqueous solution of apomorphine, injected rapidly into the saphenous vein. Tests were performed never more often than every third day on any one animal. The dog was fed a ration of horsemeat a few minutes before the experiment and nothing short of the actual expulsion of vomitus was considered a positive reaction to the injected apomorphine. Ten μg/kg apomorphine was used as the first dose; if this was effective, the dose was reduced to 7.5 or 5 μg/kg. On the other hand, if emesis did not occur following the initial dose of apomorphine, it was increased to 20 μg/kg. The threshold emetic dose in each animal was determined on the basis of at least 3 successive positive results. To test for anti-emetic activity of Benadryl and Dramamine, these agents were administered intravenously 15 min prior to the threshold dose of apomor-

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† Medical student, University of Utah; research conducted in partial fulfillment of requirements for course work in pharmacology.

‡ Dramamine was supplied by G. D. Searle & Co.

§ Benadryl was supplied by Parke Davis & Co.

TABLE I. Determination of Emetic Threshold to I.V. Apomorphine in 5 Dogs.

.005 mg/kg			.0075 mg/kg			.01 mg/kg			.02 mg/kg		
Latent period, min.	sec.	Paroxysms	Latent period, min.	sec.	Paroxysms	Latent period, min.	sec.	Paroxysms	Latent period, min.	sec.	Paroxysms
	*			—	0	2	30	1	1	20	3
							—	0	1	40	3
						1	5	4	1	30	4
	*			—	0		—	0	1	30	1
						1	30	2	1	10	1
									1	20	1
—		0		—	0	1	0	2	1	40	3
							—	0	1	30	1
						2	0	1	1	30	1
—		0	0	45	4	0	40	2		*	
			0	45	3						
			0	45	3						
2	0	1	1	50	1	1	25	3		*	
—		0	2	0	1						
			1	15	1						

* Not tested.

TABLE II.
Effect of Dramamine and Benadryl on Apomorphine-Induced Emesis in 5 Standardized Dogs.*

Threshold dose of apomorphine, mg/kg	Latent period of vomiting induced by threshold dose of I.V. apomorphine: Control, min.	sec.	After I.V. Dramamine dose: 2 mg/kg, min.	sec.	5 mg/kg, min.	sec.	10 mg/kg, min.	sec.	After I.V. Benadryl dose: 1 mg/kg, min.	sec.	2.5 mg/kg, min.	sec.
.02	1	30	1	20	1	50		†	1	30	0	40
.02	1	20	0	50	1	50	1	30	1	0	1	5
.02	1	35	1	20	1	10		†	1	30	1	35
.0075	0	45	0	35		†	1	0	1	15		†
.0075	1	40	2	5	1	25		†	1	15	1	0

* Same dogs and order as in Table I. † Not tested.

phine. *Additional experiments* were performed on dogs and cats in the attempt to reproduce certain results, reported in the literature, obtained with the above-mentioned drugs. The methods described in these reports were duplicated as faithfully as possible. The specific technics employed are outlined in the appropriate discussions below.

Results. The results of the individually determined emetic thresholds for *i.v.* apomorphine in dogs are shown in Table I. The average latent period of vomiting was 1.5 min. These animals were subsequently injected intravenously with Dramamine or Benadryl to detect any inhibition of the emetic response to the threshold dose of *i.v.* apomorphine (see Table II.) Doses of 2 and 5 mg/kg of *i.v.* Dramamine caused no detectable inhibition of the vomiting response to apomorphine or increase in the latent period of emesis. Dramamine itself produced vomiting, ataxia and excitation in doses of 10 mg/kg. Nevertheless, dogs treated with this high dose of Dramamine again vomited following the threshold dose of apomorphine. With doses of 1 and 2.5 mg/kg *i.v.* Benadryl, there was no apparent difference in the incidence or the character of the apomorphine-induced emesis as compared with the controls.

The experiment of White and coworkers (5), on the protective effect of Dramamine against apomorphine-induced emesis, was repeated in 4 dogs. The control dog, untreated with Dramamine, vomited 2 min after the subcutaneous injection of 30 mg/kg apomorphine. Subsequently, the animal showed

TABLE III. Effect of Oral Dramamine and Benadryl on Latent Period of Vomiting Induced by Subcutaneous Apomorphine in Cats.*

Apomorphine control, min.	Apomorphine given 15 min. after		Apomorphine given 60 min. after	
	Dramamine, min.	Benadryl, min.	Dramamine, min.	Benadryl, min.
2	8	24	16	8
7	5	18	30	17
6	4	16	10	25
			15	7
Avg 5	6	19	18	14

* Dramamine and Benadryl: 75 mg, *per os*. Apomorphine: 65 mg, s.c.

hyperexcitability and continuous running, and it recovered only after several hours. The remaining 3 dogs received Dramamine, 2 mg/kg in 40 ml of water by stomach tube, 1 to 1.5 hours before the 30 mg/kg dose of apomorphine. Following the apomorphine, they vomited and showed the above-mentioned toxic reactions. No protective effect of Dramamine was demonstrated. We attempted to reproduce the experiments of Mitchell(8) who reported that Dramamine caused a greater inhibition of apomorphine-induced vomiting in cats than did Benadryl. The results obtained in 7 cats are shown in Table III. Apomorphine, 65 mg in 2.5 ml of water, was given subcutaneously 15 min after the oral administration of 75 mg Dramamine or Benadryl in 30 ml of water. The control untreated cats vomited after an average latent period of 5 min following the injection of apomorphine. Dramamine did not prevent emesis and there was no significant difference between the average latent periods in the control and the Dramamine-treated cats. Benadryl likewise did not prevent the vomiting. However, Benadryl did prolong the average latent period of emesis approximately 4-fold over the control value. Because 15 min might have been insufficient for adequate absorption from the gastrointestinal tract, another series of experiments was performed in which one hour was permitted to elapse between the oral administration of Dramamine or Benadryl and the injection of subcutaneous apomorphine. In spite of this precaution, no prevention of emesis resulted. However, Dramamine prolonged the latent period of emesis to approximately the same extent as did Benadryl. To rule out the disturbing factor of the development of tolerance to apomorphine, reported by Mitchell(8), some of the experimental cats were tested with apomorphine alone at the end of the investigation. These animals vomited after latent periods which approximated their original control values.

In order to determine the effects of the "anti-emetics" on the vomiting initiated by a different type of emetic stimulus, cats treated with *i.v.* Dramamine were tested with oral copper sulfate. It was first established that the dose of 20 mg of copper sulfate in 25 ml of water was effective in eliciting emesis in all of 14 normal fasted cats. Dramamine, 5 mg/kg, given intravenously 15 min prior to this oral dose of copper sulfate, failed to prevent vomiting in all of four cats tested. The average latent period of vomiting was 35 min, which compares favorably with control values.

Discussion. The advantages of determining the intravenous threshold emetic dose of apomorphine for each animal are as follows: Vomiting is elicited consistently with a reproducible latent period; development of tolerance is avoided; a minimal stimulus is provided; and the problem of sex difference in apomorphine susceptibility, claimed by Chen and Ensor(3) to occur with the subcutaneous route, is circumvented. Despite the use of this sensitive emetic test, no inhibition of apomorphine-induced emesis was obtained with either Dramamine or Benadryl.

Two possible sites of an anti-emetic action of Dramamine and Benadryl are a) the medullary emetic chemoreceptor trigger zone in the area postrema(9) and b) the vomiting center in the lateral reticular formation of the medulla. If the emetic effect of centrally-acting drugs, such as apomorphine and *i.v.* cardiac glycosides(10), is prevented by an anti-emetic agent without a concomitant reduction in re-

sponsivity to peripherally-acting emetics, such as oral copper sulfate(11), then the site of the anti-emetic action would be localized to the chemoreceptor trigger zone and/or its pathway to the emetic center. On the other hand, if both centrally- and peripherally-acting emetics become inactive after anti-emetic therapy, then a direct inhibitory action on the emetic center itself would be a sufficient explanation for this effect, although receptor sites and afferent pathways might be blocked as well. Unfortunately, the lack of anti-emetic activity of Dramamine and Benadryl in the present series of experiments makes localization of their site(s) of action impossible. However, if prolongation of the latent period of drug-induced vomiting can be considered to constitute a degree of protection, then some interpretation of the results becomes feasible. By the oral route Benadryl was found to be more effective than Dramamine in prolonging the latent period of vomiting in response to *s.c.* apomorphine in the cat, when 15 min were allowed for absorption from the gastrointestinal tract, but the two drugs were equally effective in prolonging the latent period when one hour was allowed for absorption. With regard to the emetic response to oral copper sulfate, no increase in the latent period was evident after *i.v.* Dramamine. On the basis of the above discussion, these findings would seem to imply that the anti-emetics studied may selectively depress the chemoreceptor trigger zone, but it must be re-emphasized that they merely prolonged the latent period without preventing the emetic response to apomorphine.

Summary. 1. Neither Dramamine (2, 5 and 10 mg/kg) nor Benadryl (1 and 2.5 mg/kg) administered intravenously was effective in preventing the emetic responses to threshold doses of intravenous apomorphine or in prolonging the latent periods of vomiting in 5 standardized dogs. 2. Other experiments were performed in the attempt to verify the reported protection afforded by Dramamine and Benadryl against apomorphine-induced emesis in dogs and cats. Although the latent period of emesis was prolonged in one of the experiments on cats, these drugs did not prevent vomiting.

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Hemorrhagic Diathesis Due to PTC (Plasma Thromboplastin Component) Deficiency.* (20142)

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Aggeler, White, Glendening, Page, Leake, and Bates(1) described for the first time a congenital hemorrhagic disease resembling hemophilia which they have called PTC (plasma thromboplastin component) deficiency. This disease is similar to hemophilia in exhibiting a prolonged coagulation time and abnormal prothrombin consumption. Prothrombin, proaccelerin (AcG or labile factor), proconvertin (pro-SPCA, factor 7, co-thromboplastin of Mann), fibrinogen and platelets are described as normal. The clotting defect differs from hemophilia in that it can be corrected by the addition of hemophilic as well as normal plasma and by the plasma factor, PTC. At the time the above-cited paper was published, we were studying a patient with a circulating anticoagulant. This patient (R.J.) resembled some of the described cases of hemophilia with circulating anticoagulants (2,3), in that he apparently suffered from a congenital hemorrhagic disease and had developed the anticoagulant subsequent to transfusion. Studies on this patient, reported here, were made at a time when the anticoagulant had almost completely disappeared and show that his basic disease was PTC deficiency and not hemophilia. The characteristics of the anticoagulant will be described in a separate communication(4).

In the last few months, we have had the opportunity to study 4 additional patients with prolonged coagulation times. Two of these, E.W. and Q.G., proved to have PTC deficiency and the others, W.B. and T.G., to have true hemophilia. These findings suggest that PTC deficiency may be a fairly common hemorrhagic disorder, previously unrecognized or confused with hemophilia.

Methods. All blood samples were carefully collected by venipuncture into silicone syringes and transferred to glass or silicone containers as desired. *Platelet "thromboplastin"* was measured by preparing washed (8x) platelet suspensions from normal and patients' citrated plasmas and observing their ability to restore the coagulation of normal platelet poor plasma (centrifuged at 20,000 *g* for 90 minutes). *Coagulation time* was measured by the Lee-White method(5) at 37°C. *Prothrombin consumption* was performed by a modification of the method of Quick(6). Blood samples were oxalated (one-tenth volume of 0.1 M sodium oxalate) after standing at 37°C for one hour after clotting. One-tenth ml of serum obtained by centrifugation was added to 0.1 ml of $BaSO_4$ plasma plus 0.1 ml thromboplastin (soluplastin, Schieffelin and Co., courtesy Dr. E. W. Blanchard). The clotting time was determined after addition of 0.1 ml 0.04 M calcium chloride. *Prothrombin concentration* was determined by the one-stage method of Quick(7), employing soluplastin, human brain thromboplastin or acetone dried dog brain obtained from a dog pretreated with dicumarol until only traces of prothrombin could be detected in his blood. The dog was anesthetized, exsanguinated and the brain was thoroughly perfused *in situ* before removal. *Labile factor* was assayed by mixing equal quantities of normal or patients' $BaSO_4$ plasmas with a standard aged plasma and comparing the reduction in "prothrombin time." *Co-thromboplastin* was assayed by the method of Mann(8). *Two-stage prothrombin* concentration tests were performed by the method of Ware and Seegers(9) modified to supply AcG. *Fibrinogen* was clotted by thrombin and determined gravimetrically. *Anticoagulant* titer was measured by a method to be described in detail(4). *Plasma antithrombin* was determined by adding 0.1 ml of thrombin (Parke, Davis and Co., courtesy Dr. E. A. Sharp) of various concentrations (10 u, 5 u, 2.5 u, 1.25 u/ml), prepared in silicone tubes to prevent rapid de-

* This investigation was supported in part by a research grant from the Division of Research Grants of the National Institutes of Health, U. S. Public Health Service.

TABLE I. Historical Data on 5 Patients.

	PTC deficiency			Hemophilia	
	E.W.	R.J.	Q.G.	W.B.	T.G.
Age	4	16	40	20	40
Sex	♂	♂	♂	♂	♂
Race	C	C	W	W	W
Age at onset of symptoms	1½	3	4	Infancy	6
Symptoms:					
Epistaxis	+	0	0	+	+
Hematoma	+	+	+	+	+
Hemarthroses	+	+	0	+	+
Hematuria	+	0	+	+	+
Relative severity of bleeding tendency	4+	4+	+	4+	2+
Previous response to blood or plasma	+	+	+	+	+
Family history	+	Neg	+	+	+
Transmission	♀		♀*	♀*	♀
Sex	♂		♂	♂	♂

* or affected ♂.

terioration, to 0.1 ml oxalated plasma and determining the clotting time; at least one normal plasma was used as a control. *Antithromboplastin, immediate,* was determined by measuring the prothrombin time, employing dilutions of human brain thromboplastin and comparing these times to those of normal plasmas. *Antithromboplastin, progressive,* was determined by mixing silicone citrated plasma with an equal volume of a one to 20 dilution of human brain thromboplastin, incubating at 37°C and measuring the clotting time obtained when 0.2 ml of this mixture was added to 0.1 ml of 0.02 M $CaCl_2$, in silicone tubes, at intervals for 15 minutes. Simultaneous tests were performed on normal control plasmas. PTC was prepared as described by Aggeler *et al.*, and was concentrated by dissolving the dialyzed, lyophilized product in a volume equal to one-tenth of the original plasma volume. Tests showed that the PTC fraction contained no thrombin or SPCA(10). It did contain an insignificant trace of prothrombin. $BaSO_4$ plasma was prepared by mixing 100 mg of barium sulfate per ml of oxalated plasma for 10 minutes at room temperature and removing the barium sulfate by rapid centrifugation. This plasma would not clot on addition of thromboplastin and calcium.

Results. Pertinent data from the histories of the 5 patients studied are presented in Table I. All the patients were male and 2 of the patients with PTC deficiency were negroes. The type of hemorrhage, severity of disease, response to transfusion and familial transmission did not differentiate between the PTC and hemophilic groups. The incidence of hemorrhagic tendency in the families of 2 of the PTC deficient patients is shown in Fig. 1. R.J.'s mother died shortly after his birth and no adequate family history was obtained.

Routine hemostatic studies showed no detectable difference between the PTC deficient

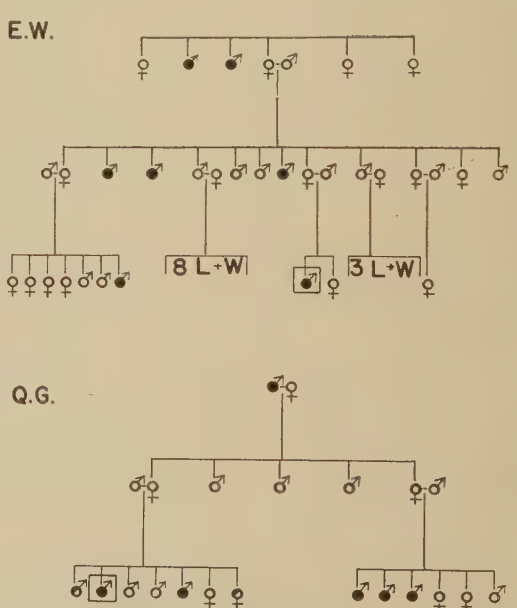

FIG. 1. Familial transmission in patients E.W. and Q.G. Open circle, no hemorrhagic history; half-closed circle, died in infancy; closed circle, hemorrhagic tendency; open square indicates patient studied.

TABLE II. Coagulation Studies.

Test	PTC deficient			Hemophiliac		Normal range
	E.W.	R.J.	Q.G.	W.B.	T.G.	
Clotting time:						
Glass	2–8 hr	>8 hr	24 min.	90 min.	24 min.	6–12 min.
Silicone	>8 hr	>8 hr	78 min.	>4 hr	4 hr	30–60 min.
Clot retraction	4+	3+	4+	3+	4+	4+
Clot lysis	0	0	0	0	0	0
Prothrombin consumption	10.6 sec.	13.8 sec.*	13.2 sec.	12 sec.	20.8 sec.	>50 sec.
Prothrombin time:	Sec.					
Soluplastin—Patient	12.4	14.4	15.2	15.0	17.5	
(Normal)	(15.0)	(15.6)	(17.9)	(15.0)	(15.0)	(15–18)
Human brain—Patient	17.4	17.3	21.2	22.3	15.3	
(Normal)	(17.1)	(17.3)	(17.5)	(19.3)	(17.4)	(17–20)
Dog brain—Patient	26.5	22.9	—	—	32.2	
(Normal)	(34.2)	(25.9)	—	—	(31.2)	(25–35)
2-stage prothrombin (u/ml)	300	276	280	—	280	260–300
Labile factor assay	100%	100%	—	—	—	100%
Co-Thromboplastin	100%	100%	—	—	—	100%
Fibrinogen (mg %)	230	440	320	380	360	250–500

* Due to the very long coagulation time, the usual method of measuring prothrombin consumption was unsatisfactory. Samples of normal and patient's blood were refrigerated 48 hr and residual prothrombin determined. Normal value was 26 sec.

and the hemophilic patients. Bleeding times, tourniquet tests and platelet counts did not differ significantly from the normal range with the exception of one prolonged bleeding time observed in patient Q.G. Plasma antithrombin and antithromboplastin titers were normal, and no anticoagulant could be detected with the exception of a trace in R.J., the patient who had previously shown a high titer. Additional clotting studies are shown in Table II. All patients showed normal clot retraction and their isolated platelets were able to restore the defect in platelet poor plasma. All patients showed prolonged coagulation times and abnormal prothrombin consumption. The one-stage prothrombin times were within the normal range even with the specially prepared dog brain thromboplastin. This finding suggested that prothrombin, proaccelerin, and proconvertin were normal in these patients. To confirm this we measured (a) the prothrombin by the 2-stage method, (b) the labile factor (proaccelerin, AcG) by observing the ability of normal and patients' prothrombin free plasmas to restore the "prothrombin time" of aged plasma, and (c) co-thromboplastin which is thought to be the same as proconvertin or SPCA precursor(11). All results were within the normal range. On one occasion attempts were made to assay SPCA in E.W. and R.J., but unfortunately the blood samples did not clot in 8 hours and tests made the following day showed only traces of SPCA activity. Normal serum incubated for a similar time at 37°C, also showed only traces of SPCA activity.

Many additional studies were done on these patients. Of particular interest are the plasma recalcification studies shown in Table III. Citrated plasma samples from all of the patients showed prolongations of their recalcification times (normal range = 2-2½ minutes). Clotting was markedly shortened in the plasmas of E.W., R.J., and Q.G. by addition of plasmas from W.B. and T.G., by normal plasma and by PTC factor. Clotting in patients W.B. and T.G. was shortened by addition of plasma from E.W., R.J., and Q.G., by normal plasma and by normal $BaSO_4$ plasma. These simple tests served to differentiate the 2 groups: the PTC deficient and the hemophilic. Normal plasma was able to restore the clotting defect in both groups. It was possible to fractionate this normal plasma into (a) the PTC fraction, which restored the defect in PTC deficiency, but did not shorten significantly the recalcification time of the hemophilic group and (b) $BaSO_4$ plasma,

TABLE III. Plasma Mixture Recalcification Times. Citrated plasmas, previously frozen in silicone, from the 5 patients were recalcified in glass, after mixture with one-fifth part of the listed reagents.

	0.2 ml citrated plasma											
	PTC deficient						Hemophiliac					
0.05 ml added reagent	E.W.		R.J.		Q.G.		W.B.		T.G.		Normal	
	Min.	Sec.	Min.	Sec.	Min.	Sec.	Min.	Sec.	Min.	Sec.	Min.	Sec.
Patient's own plasma	8	30	26	00	6	30	18	50	10	10	2	15
Saline	8	00	26	00	6	30	18	20	10	20	2	10
Plasma:												
E.W.		—	26	00	6	00	3	50	3	55	2	05
R.J.	17	30		—	7	30	4	00	4	00	2	05
Q.G.	6	30	20	30		—	3	50	3	55	2	05
W.B.	3	15	6	15	3	15		—	10	35	1	40
T.G.	2	50	7	00	3	20	13	30		—	1	55
Normal	2	55	7	30*	3	20	4	10	4	30		—
$BaSO_4$ plasma	8	10	26	00	6	30	4	40	4	25	2	15
PTC factor	1	40	3	20	1	55	12	30	7	10	1	50

* This figure suggests that R.J.'s plasma still contains a trace of anticoagulant.

which shortened the hemophilic clotting but not the PTC deficient.

A number of PTC fractions, free from SPCA and thrombin, were prepared both from plasma and from serum. Freshly prepared serum fractions were usually more active than plasma fractions, but after prolonged dialysis and drying, the 2 types of PTC showed equal activity.

Discussion. In considering the differential diagnosis of a hemorrhagic diathesis manifesting a prolongation of the whole blood coagulation time, the following possibilities must be considered: 1) hemophilia, 2) PTC deficiency, 3) qualitative or quantitative platelet deficiency (the Lee-White coagulation time is occasionally prolonged in severe deficiencies), 4) hypoprothrombinemia, 5) hypoproconvertinemia (deficiency of co-thromboplastin or SPCA precursor), 6) hypoproaccelerinemia (parahemophilia, deficiency of labile factor or plasma AcGlobulin), 7) hypo- or afibrinogenemia, and 8) circulating anticoagulant. The experimental data presented in Table II eliminate the last 6 possible diagnoses and suggest that the differential diagnosis lies between hemophilia and PTC deficiency. As Aggeler *et al.* have shown, this differentiation may be made by studying the ability of $BaSO_4$ normal plasma and the plasma fraction, PTC, to restore the coagulation defect. The plasma mixture recalcification test (Table III) proved a simple method for obtaining this differentiation.

The exact role of PTC in the coagulation mechanism has not been defined. Aggeler *et al.* chose the name PTC (plasma thromboplastin component) because tissue thromboplastin can substitute for or by-pass the role of PTC in clotting.

Summary. Five patients suffering from hemorrhagic diatheses manifesting prolongation of the whole blood coagulation time are presented. On the basis of the experimental evidence, 3 of these patients have been classified as PTC deficient and 2 as hemophilic.

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Prothrombin Activity of Blood of the Newborn.*† (20143)

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It has been repeatedly observed that the blood of the newborn baby has a normal prothrombin time, provided the mother is supplied with vit. K before the baby is born, but a prothrombin concentration of only about 30% when measured by the two-stage method. Brinkhous, Smith and Warner(1) originally explained this discrepancy by postulating a compensatory acceleration of the conversion of prothrombin in newborn blood. More recently this acceleration is being attributed to specific conversion factors(2). If this explanation is valid, the plasma of a newborn baby should contain a relatively high concentration of the accelerator and a low amount of prothrombin, whereas normal adult blood should have 3 times more prothrombin and a corresponding lower concentration of the accelerator. If the prothrombin time is dependent not only upon prothrombin, but also on the accelerator factor, one should expect that when adult and newborn baby plasmas are mixed in varying proportions a detectable change in prothrombin time should occur since the ratio of prothrombin to accelerator can be greatly varied. From the results recorded in Table I it is clear that in a series of mixtures ranging from a ratio of 9 parts of newborn to 1 part of adult plasma to a reverse ratio of 1 to 9, no demonstrable change in the prothrombin time was observed.

To obtain further information concerning the relative prothrombin activity of newborn plasma, the effect of storage in glass was investigated. When normal adult plasma is stored at 4°C in glass, the prothrombin time which initially is 12 seconds becomes prolonged due to loss of labile factor. On restoring the latter by adding deprothrombinized rabbit plasma which serves as a rich source, the prothrombin time becomes shortened to about 8 to 9 seconds. The prothrombin time of newborn plasma likewise becomes prolonged on storage, but restoration of the labile factor brings the prothrombin time only to 12 seconds. When stored plasmas of the newborn and of the adult were mixed in varying proportions and deprothrombinized rabbit plasma added, the prothrombin times of the mixtures corresponded with the values expected on the basis of the prothrombin curve(3).

It is experimentally established that a decrease of either prothrombin or labile factor prolongs the prothrombin time. Since the concentration of labile factor is approximately the same in newborn as in adult plasma(4), it is unlikely that the agent is the accelerator hypothesized to explain the observed normal prothrombin time. The present results furnish no support for an accelerator, and a search of the literature fails likewise to disclose any convincing evidence that such a compensating converting factor exists in newborn blood. Such an agent was postulated to account for the seemingly paradoxical situation of a normal prothrombin time with a prothrombin as measured by the 2-stage procedure of less than one-third of normal. By correlating the present results with the previous findings(4) that newborn blood has the same concentration of active prothrombin as adult blood, but contains little or no inactive prothrombin (prothrombinogen), which in adult blood constitutes the largest fraction of the total prothrombin, the discrepancy in findings between the one and two stage methods is readily explained without introducing hypothetical accelerator or conversion factors.

Summary. When adult and newborn plasmas were mixed in varying proportions, the prothrombin time which was 12 seconds for each plasma remained unaltered in the series of mixtures. This suggests that the prothrombin activity *i.e.*, the concentration of active prothrombin, is the same in the plasma of the

* Cord bloods were obtained through cooperation of the Department of Obstetrics, Milwaukee Hospital.

† This investigation was supported by a grant from the Division of Research Grants, National Institute of Health, USPHS.

TABLE I. The Effect of Mixing Plasmas of Newborn and Adult on Prothrombin Time.

Fresh cord plasma	cc	.1	.09	.08	.07	.06	.05	.04	.03	.02	.01	.0	
" adult "	cc	—	.01	.02	.03	.04	.05	.06	.07	.08	.09	.1	
Prothrombin time*	sec.	12	12	12	12	12	12	12	12	12	12	12	
Stored cord plasma	cc	.1	.1	.09	.08	.07	.06	.05	.04	.03	.02	.01	—	—
" adult "	cc	—	—	.01	.02	.03	.04	.05	.06	.07	.08	.09	.1	.1
Deprothrombinized rabbit plasma	cc	—	.02	.02	.02	.02	.02	.02	.02	.02	.02	.02	.02	—
Prothrombin time*	sec.	24	12	10½	10	9½	9	8½	8½	8¼	8	8	8	35

* The method employed has been previously described(3).
The mothers were supplied with vit. K (synkayvite) prepartally.

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Growth of Poliomyelitis Viruses in Large Stationary Cultures.* (20144)

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The first reported methods for propagating poliomyelitis viruses in tissue cultures utilized implants of the suspended cell type(1). Subsequently roller tubes in which the proliferating fragments of tissue were imbedded in plasma clots were used(2). In addition to permitting direct microscopic visualization of cultures for cytopathogenic effect, this method had the advantage of giving an early growth of virus(3). Recent reports have indicated that these same advantages obtain if culture tubes are kept stationary instead of rotated(4,5).

In this report are presented results obtained with the growth of poliomyelitis viruses in large stationary flask cultures. A simplified medium (Hanks-Simms) and fragments of monkey testes imbedded in plasma clots were used. The work was done in order to obtain large quantities of supernatant fluid of sufficiently high virus titer for complement-fixing antigen preparation according to the method of Svedmyr and Enders(6) without having to maintain many small culture units and without having to use special equipment for rotating large culture bottles.

Materials and methods. Viruses. Brunhilde (type 1), Y-SK (type 2) and Saukett† (type 3) strains of poliomyelitis viruses were used in titered pools of supernatant fluid from infected roller tube cultures of monkey testes. Each virus strain had been through at least three consecutive tissue culture passages, and was specifically neutralized by prototype antiserums. *Chicken plasma.* Dehydrated chicken plasma (Difco) was reconstituted and carbonated in the manner reported by Melnick (7). *Monkey testes.* Testes from 3 to 5 normal immature *rhesus* (*M. mulatta*) monkeys were minced together and used for cultures on the same day they were obtained. *Nutrient fluid.* Two kinds of fluid were used. One referred to as "complete medium" contained Hanks and Simms solutions and acetone-extracted chick embryo extract(7). The

* Aided by a grant from the National Foundation for Infantile Paralysis.

† We are indebted to Dr. Jonas Salk for supplying us with the Saukett strain.

FIG. 1. Culture in Roux flask illustrating the amount and distribution of tissue.

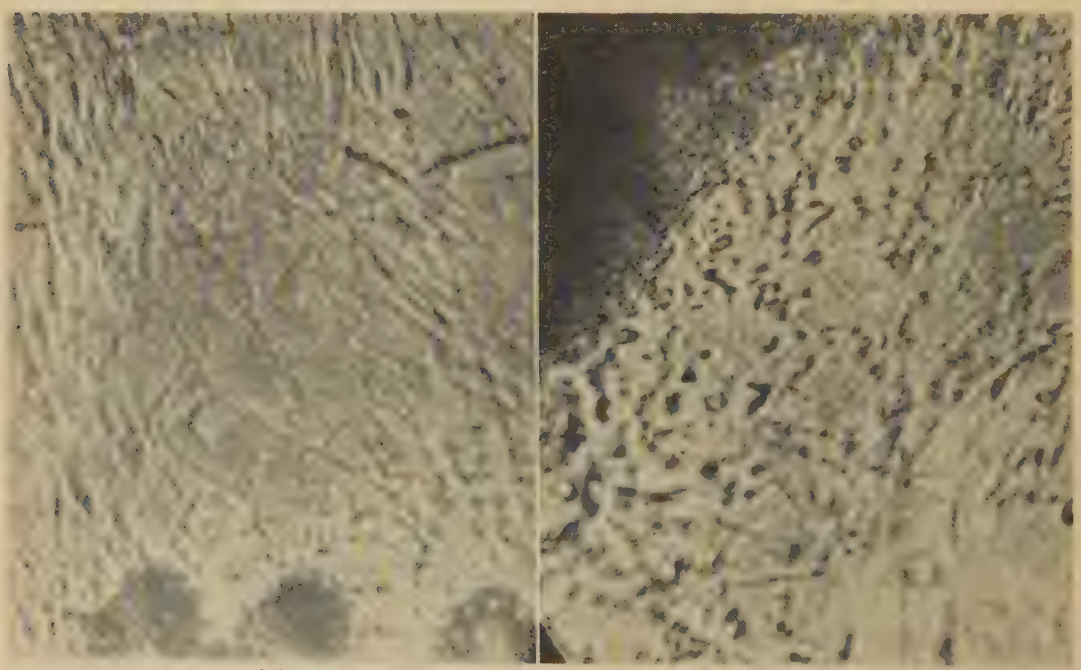
complete medium was used for all fluid changes in roller tube cultures and for fluid changes in flask cultures during the preinoculation growth phase. Another medium referred to as "simplified medium" contained only Hanks and Simms solutions in ratio of 3:1. The simplified medium was used for fluid changes in flask cultures at the time of virus inoculation and thereafter. All nutrient fluids contained 50 units of penicillin and 50 μg of streptomycin per ml.

Growth of virus. a) *In 125 ml Erlenmeyer flasks.* The necks of 125 ml Erlenmeyer flasks were bent so that the plane of the flask mouth was vertical in order to minimize the risk of air-borne contamination. The flasks were cleaned and sterilized according to described procedures(7) for tissue culture use. Reconstituted chicken plasma, 0.25 ml, was spread by agitation over the bottom of each flask. A fine mince of monkey testes was distributed in the plasma film with a pipette until about 10-12 fragments of tissue were contained in each square centimeter on the bottom of the flask. The plasma was clotted with 0.2 ml of chick embryo extract. After half an hour 10 ml of complete medium were added. The flasks were stoppered with gauze-covered cotton, sealed with parafilm‡ and incubated at 35°C. Nutrient medium was changed every 3 days. Simplified medium was used for the second fluid change and for all subsequent ones. The virus to be tested for growth (100 or 1000 roller tube infecting doses) was added with the second fluid change. All supernatant fluids after that were saved and stored at –70°C. The cultures were discontinued one month after the virus had been added. b) *In Roux flasks.* Roux culture bottles with a capacity of 1000 ml were cleaned and sterilized. One ml of reconstituted plasma was spread by agitation over one side of the flask. A fine mince of monkey testes was distributed with a long pipette through the plasma film until there were about 8-12 fragments per square centimeter (Fig. 1). One ml of chick embryo extract was added to clot the plasma. After half an hour 75 ml of complete medium were added, the flask was stoppered with gauze-covered cotton, sealed with parafilm and incubated at 35°C. For the first fluid change complete medium was used. Simplified medium was used when the virus was introduced at the second fluid change and for all changes thereafter. After virus inoculation (750 roller tube infecting doses) all supernatant fluids were saved and stored at –70°C. Cultures were discontinued thirty days after virus inoculation. c) *In roller tubes.* Roller tube cultures were prepared in the manner reported by Melnick(7). Supernatant fluids were replaced with complete medium when the tubes were three and five days old. Virus was

‡ Parafilm may be obtained from the Marathon Corporation, Menasha, Wis.

FIG. 2. Cellular proliferation from an implant of monkey testis in Roux flask culture, 6 days old. Microscopic detail of culture was quite poor because of the two thick layers of irregular glass, through which light was transmitted.

added at the time of the second fluid change. Fluids were changed every three days thereafter. The supernatant fluids were obtained and stored at −70°C. Later, virus growth curves were determined. *Titrations.* Supernatant fluids containing poliomyelitis virus were diluted serially in half logarithm increments (serial 3.2 dilutions) with Hanks solution. Each dilution was tested in 0.1 cc amounts in two 5-day-old roller tubes. The



(a) (b)

FIG. 3. (a) Cellular proliferation in a Roux flask culture at 12 days without virus inoculation. (b) Twelve-day-old Roux flask culture showing cytopathogenic effect after Brunhilde virus inoculation 6 days previously.

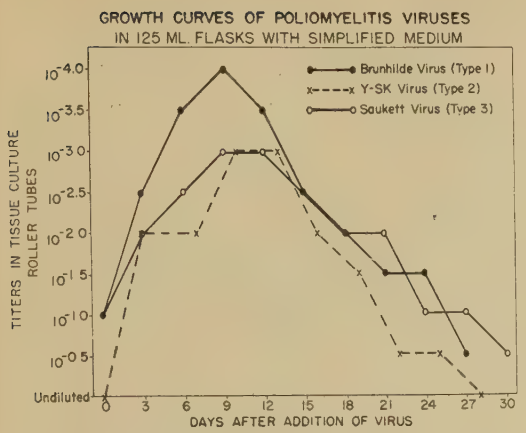


FIG. 4. Titers at zero days indicate virus concentration in flask fluids immediately after adding the virus inoculum.

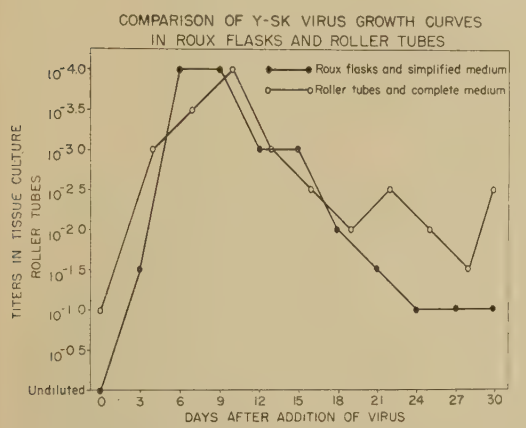


FIG. 5. Titers at zero days indicate virus concentration in flask fluids immediately after adding the virus inoculum.

tubes were read for cytopathogenic effect 6 days after inoculation (time of the second post-inoculation fluid change). The endpoint was taken as the greatest dilution which caused a cytopathogenic effect in both tubes. The dilution of virus that occurred in the roller tubes at the time of inoculation was not taken into account in figuring final titers.

Results. Cultures in 125 cc Erlenmeyer flasks. Brunhilde, Y-SK and Saukett strains of poliomyelitis viruses were successfully propagated. With each strain, supernatant fluids from two or more consecutive fluid changes yielded titers of 10^{-3} or more in roller tube titrations (Fig. 4). Titers above 10^{-2} were obtained in supernatant fluids from 5 to 7 consecutive fluid changes. Maximum growth occurred between the third and eighteenth days. The highest titers were obtained 9 or 10 days after virus inoculation.

Cultures in Roux flasks. At the time of virus inoculation, when cultures were 6 days old, outgrowth of new tissue (Fig. 2) was comparable to that seen in roller tubes. Typical cytopathogenic effect occurred after virus inoculation (Fig. 3).

A growth curve was completed for Y-SK virus (Fig. 5). Virus titers greater than 10^{-3} were demonstrated for 4 consecutive supernatant fluid harvestings. Maximum growth occurred between the third and eighteenth days. The growth curve followed very closely that of the same virus strain in roller tubes with complete medium (Fig. 5).

Growth curves for Brunhilde and Saukett strains were not determined in Roux flasks. These strains multiplied and caused a cytopathogenic effect in the Roux flask cultures.

Discussion. The Erlenmeyer flask cultures were made as a pilot study for virus propagation. They were used to prepare small standard pools of virus. Their principal value was to indicate that stationary flask cultures might be used to prepare large amounts of fluid which contained virus in appreciably high titer. When the method was extended to the 1000 ml Roux flasks it was found that virus titers in supernatant fluids compared favorably with those obtained from roller tube cultures. In 15 days a single Roux flask culture infected with Y-SK virus provided 300 ml of supernatant fluid with a virus titer beyond 10^{-3}. Comparable yields with roller tubes required 75 cultures.

The use of a simplified medium appeared to make little difference as far as virus yield was concerned. Growth curves of Y-SK virus in Erlenmeyer flask cultures were compared using complete versus simplified medium. The duration and degree of maximum growth were identical. The only difference occurred in the fourth week after virus inoculation when fluid from the flasks containing complete medium gave a virus titer one logarithm higher than fluids from flasks containing simplified medium(8). The difference in titer between roller tube and Roux flask fluids, which occurred

during the fourth week of virus growth, may be the effect of different mediums rather than different culture technics.

Size of a culture unit did not appear to be a limiting factor in striving for large yields of virus. Cultures were successfully grown in six-liter rectangular culture bottles. These extremely large flasks were abandoned in favor of the Roux flasks, which were more manageable and not as prone to contamination with molds.

Summary. Poliomyelitis viruses were propagated in large stationary tissue cultures of plasma imbedded monkey testes. Large volumes of supernatant fluids with high virus titers were achieved. The virus yield, as measured by titer and duration of maximum growth, was almost identical with that obtained in roller tubes, and greater than that reported for suspended cell flask cultures. The method had another advantage in that equipment for rotating bottles was not required. Since the Roux flasks could be stacked one on top of another, a minimum of incubator space was required.

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Electron Microscopic Examination of Inclusion Bodies of Herpes Simplex Virus. (20145)

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Despite extensive investigation(1) there is still doubt concerning the relation of herpes simplex virus to the intranuclear inclusion bodies which are characteristically seen in infected tissues. Recent studies with the light microscope of such inclusions stained by the Feulgen method suggest that they contain virus and do not represent solely an abnormal product of cellular metabolism(2,3). This paper reports preliminary observations made with the electron microscope of thin sections of cells infected with herpes simplex virus.

Materials and methods. Chick embryo chorioallantoic membranes heavily infected with the HRE strain of herpes simplex virus (4) were ground with sterile sand in a mortar and suspended in nutrient broth containing penicillin and streptomycin. After centrifugation to remove large tissue particles, the supernatant fluid was diluted to contain approximately 300 ID_{50}/ml. Two-tenths milliliter of this suspension was inoculated onto the chorioallantoic membranes of 11-day-old chick embryos. Following 48 to 72 hours incubation at 35°C the membranes were removed and immediately fixed in 1% buffered osmium tetroxide, according to the method of Palade (5). Small pieces, showing pocks grossly, were excised from the fixed membrane, dehydrated in ethyl alcohol, embedded in methacrylate, and sectioned by the thermal expansion method. The sections were mounted on formvar screens and the methacrylate was removed by immersion in amyl acetate. After light shadowing with palladium the preparations were examined in an RCA type EMU electron microscope. Adjacent portions of membrane prepared by routine methods and

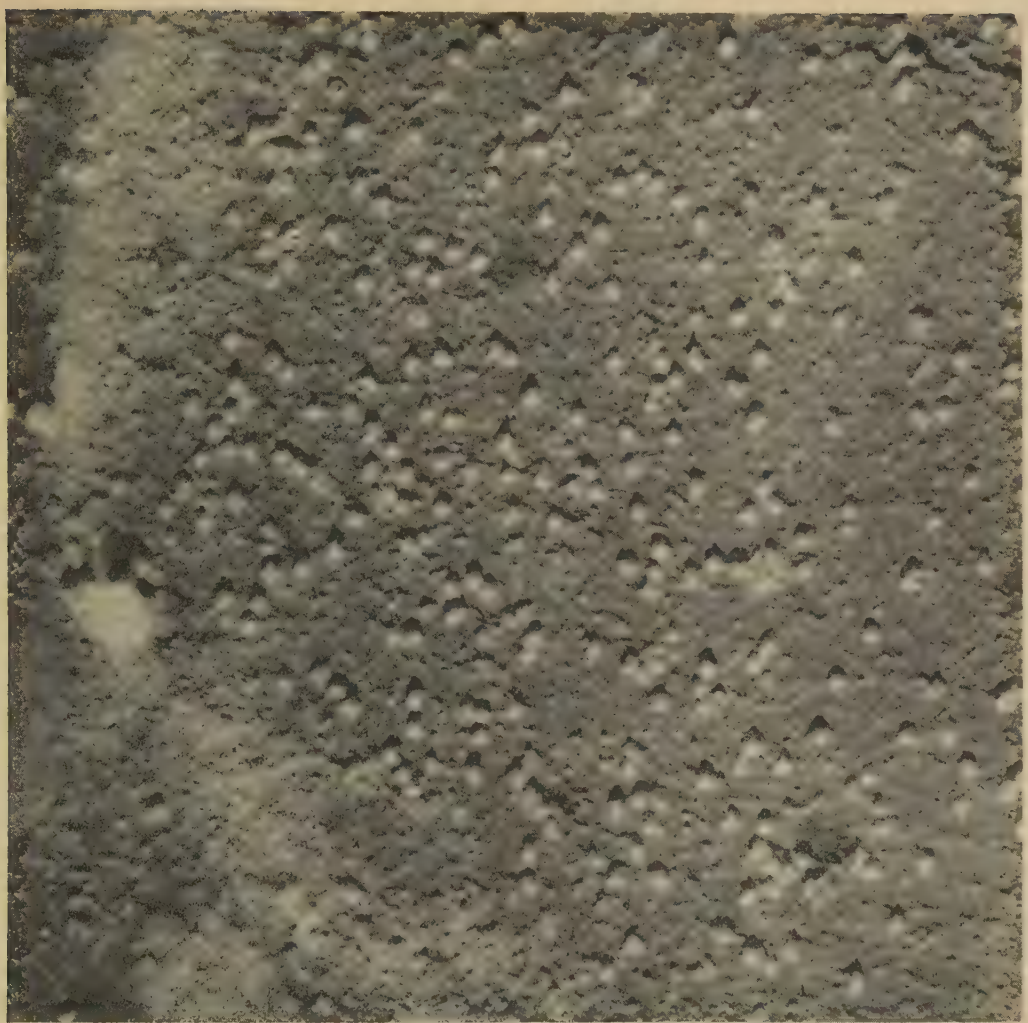

FIG. 1. Part of an inclusion body containing numerous spherical particles. Cytoplasm of host cell lies at the left. This and the following are negative prints. × 19500.

stained with hematoxylin and eosin showed numerous typical inclusions when viewed with the light microscope.

Results. The nuclei of many ectodermal cells of the chorioallantoic membrane were found to be replaced by large oval or spherical bodies readily visible in the electron microscope. Although the size of these bodies varied according to the plane of transection, they resembled both in shape and in position within the cell the inclusion bodies seen in the light microscope.

Fig. 1 shows part of an inclusion. Its border runs from the upper left hand corner downward and across the bottom. The cytoplasm of the cell is at the left margin of the picture. Distributed at random through the finely granular structure of the inclusion body are small, dense, sharply demarcated, spherical particles of nearly uniform size. Although the particles are usually dispersed through the inclusion in this fashion, occasional closely packed clusters are seen. Fig. 2 shows the margin of such an inclusion at somewhat higher magnification. The cytoplasm of the cell occupies the lower left hand corner. Clusters of particles replace the wall of the inclusion at the upper left and are partly embedded within the wall at the lower right. Some ring or shell forms are present. A few

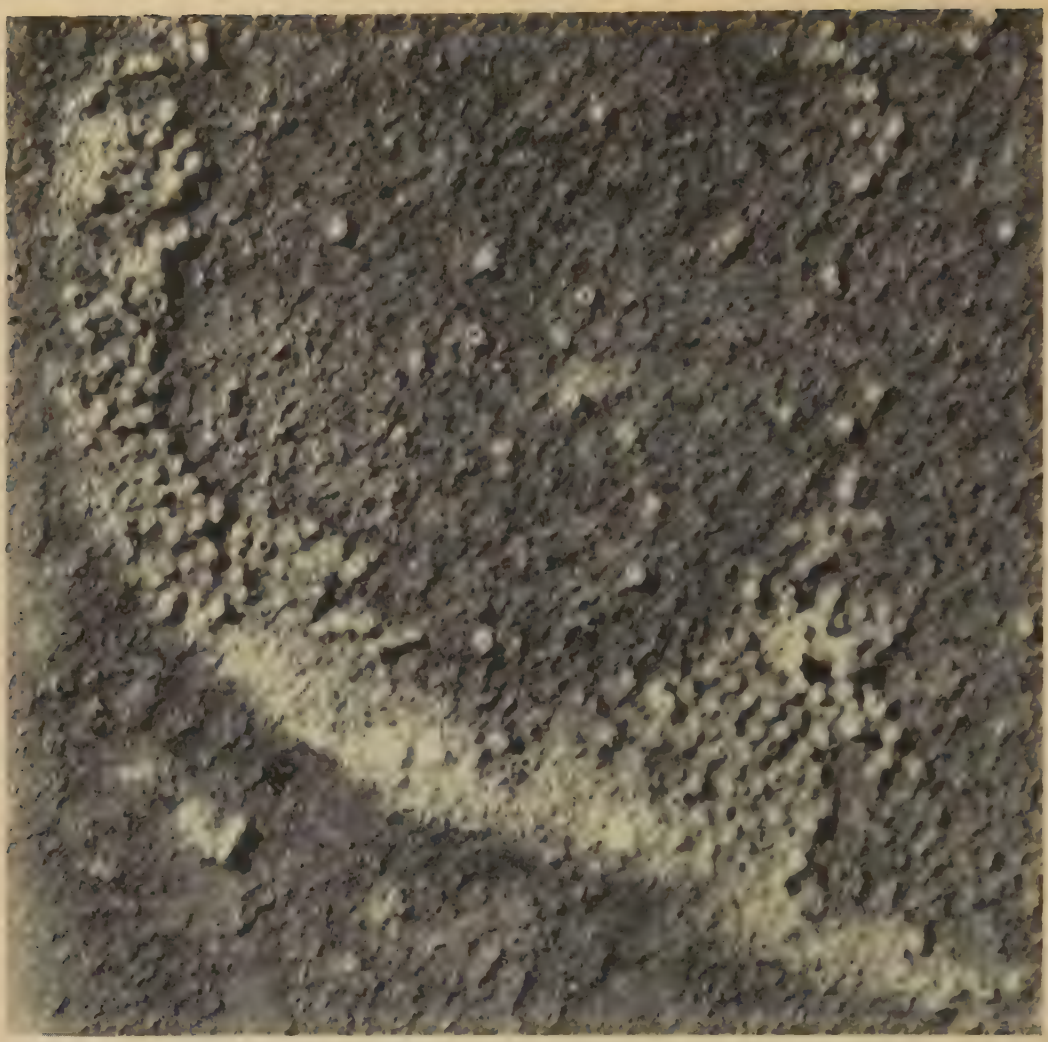

FIG. 2. The wall of an inclusion body containing clusters of particles. Cytoplasm is at the lower left corner. × 22800.

particles lie singly within the substance of the inclusion body. Sections examined before removal of the methacrylate showed inclusions containing similar particles. Most of the particles measured between 120-125 mμ, although a few ranged from 100 to 130 mμ.

Within the inclusions so far examined the number of particles has varied considerably. This is probably due in part to differences in thickness of the sections, but may also represent stages in the development of the inclusion body. Larger particles similar in size to those described by Coriell *et al.* (133-233 mμ)(6), and Evans and Melnick (200-220 mμ)(7) have been seen within the cytoplasm and at the surface of infected cells. The manner in which the inclusion body becomes differentiated and the relation of the particles within the inclusion to the larger forms seen outside are being investigated.

Summary. Intranuclear inclusion bodies were readily identified by the electron microscope in thin sections of chorioallantoic membranes infected with herpes simplex virus. Such inclusions contained numerous particles of uniform density, shape and size presumed to be elementary bodies of the virus. The particles observed within the inclusions were

smaller than other particles, also considered to be virus, seen in the cytoplasm and at the surface of cells.

The technical assistance of Miss Annette Dietsch is gratefully acknowledged.

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Pulmonary Tumors in Strain A Mice Exposed to Mustard Gas. (20146)

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Sulfur mustard and nitrogen mustards have been shown to be carcinogenic when injected into mice and rats. In tests in this laboratory(1,2) both the nitrogen mustard, methyl bis (2-chloroethyl) amine hydrochloride and sulfur mustard, bis (2-chloroethyl) sulfide, when injected intravenously into strain A mice increased the incidence and average number of pulmonary tumors to an extent comparable with that Shimkin and McClelland(3) had observed from approximately the same amount of methylcholanthrene. Boyland and Horning (4) injected stock mice subcutaneously with the nitrogen mustards, methyl bis and methyl tris, and of 14 that survived 250 days, 10 had tumors of a variety of types, one of which was a spindle-cell sarcoma at the site of injection. In this laboratory(5) tumors including sarcomas that were probably fibrosarcomas, sarcomas that were neurogenic in origin, a rhabdomyosarcoma, papillomas, and a squamous-cell carcinoma were induced in mice of strains A, C3H, and $C3H_f$ at the site of subcutaneous injection of sulfur mustard and of the nitrogen mustard, methyl bis. Griffin *et al.*(6) have reported a variety of tumors in both mice and rats injected with methyl bis and with methyl tris.

From these results, particularly those in respect to the induction of pulmonary tumors, it seemed desirable to ascertain whether or not the fumes from any of these mustards would be carcinogenic. An experiment was, therefore, set up to test the fumes of sulfur mustard, bis (2-chloroethyl) sulfide.†

Methods. Mice of strain A were used. This is a highly inbred strain that because of its high genetic susceptibility to pulmonary tumors has been used extensively for testing the carcinogenic action of chemicals particularly when injected intravenously. The incidence of spontaneous pulmonary tumors in the strain is approximately 90% in animals 18 months of age and 50% in animals 12 months of age(7). One hundred sixty individually identified strain A mice (80 males and 80 females) from 2 to 3 months of age were divided equally into control and experimental groups with littermates distributed among the 2 groups. Mice of the experimental group were placed in small individual cages of 5/8″ mesh wire and in lots of 8 were placed in an 8 liter desiccator. One hundredth cc sulfur mustard absorbed on a strip of filter paper to permit maximum evaporation was suspended in the desiccator which was immediately sealed. A small electric fan in the bottom of the desiccator circu-

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† Provided by the Medical Division, Army Chemical Center, Md.

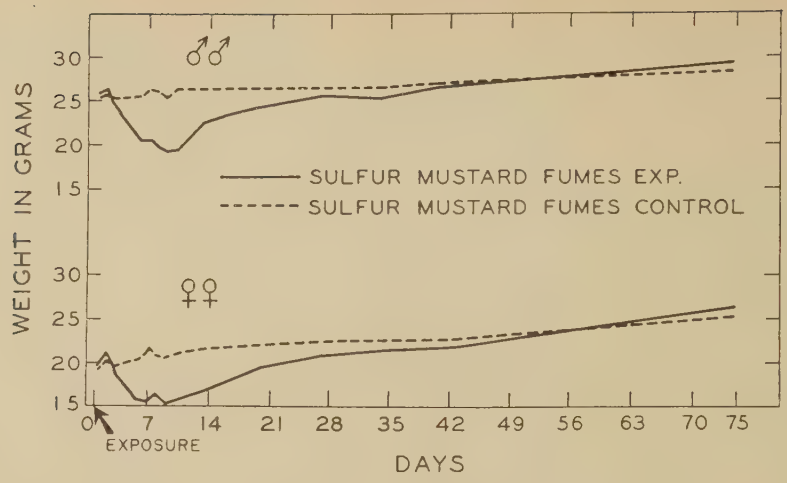

FIG. 1. Weight curves of exposed and control mice.

lated the air through the individual cages of mice during the exposure. Each lot was thus exposed for 15 minutes. Between lots the desiccator was thoroughly aired out and a new supply of sulfur mustard was suspended on a fresh filter paper. The control mice were likewise placed in individual wire cages and in lots of 8 were placed for 15 minutes in a clean sealed 8-liter desiccator. The experimental and control mice were then placed under observation in plastic cages with 8 mice to the cage and given an unlimited supply of Derwood food pellets and tap water. Sixteen males and 16 females of the experimental group and an equal number of the controls were weighed at the time of exposure, each day afterwards for 2 weeks, and subsequently at weekly intervals.

Results. The immediate response to the exposure to the mustard gas was a burning of the skin, particularly in the bare areas of the ears and nose and around the eyes. Following the first day there was also a marked reduction of weight of the exposed animals for approximately 10 days (Fig. 1). The animals then began to gain weight reaching that of the controls at approximately 8 weeks after the exposure. Thirteen mice of the experimental group died from the immediate effects of the exposure or were found dead at a later time and were too autolyzed for satisfactory examination. Three of the controls died during the course of observation and were eaten by their cage mates or were also too autolyzed for satisfactory examination.

Four months after the exposure sample lots of 30 experimental and 32 control mice were killed and examined for tumors. Nine of the experimental and 6 of the control animals had pulmonary tumors, but since the tumors of the exposed mice as well as those of the controls were small, it was decided to wait for a longer period of time before examining the rest of the animals. During the 5- to 10-month period animals were examined when they were found dead or were killed because they were moribund. At 11 months after the exposure all remaining animals of both groups were killed and examined. All tumors were fixed in Fekete's modification of Tellyesniczky's fluid,‡ sectioned and stained with hematoxylin and eosin for histologic examination. The occurrence of pulmonary tumors in the experimental and control groups is recorded in Table I. Since the 2 groups were comparable with respect to average age, incidences determined for the total of each group can be compared. Results of both sexes are combined since there was no sex difference. Of the 67 mice exposed to mustard gas 33 or 49% had pulmonary tumors as compared with 21 or 27% of the 77 control mice. This difference is highly significant; $X^2 = 7.386$;

‡ 70% ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part.

TABLE I. Occurrence of Pulmonary Tumors in Strain A Mice Exposed to Mustard Gas. No. with tumors/No. examined.

	Months after exposure								% of total with tumors	Avg age, mo
	4	5	6	7	8	9	10	11		
Experimental	9/30	1/1	1/2	0/0	0/1	0/0	2/4	20/29	49	7.5
Control	6/32	0/2	0/0	0/2	0/2	2/5	3/9	10/25	27	7.5

$P < .01$. Of the 33 of the experimental group with tumors, 24 had single nodules, 7 had 2 nodules each, and 2 had 3 nodules each. All of the 21 controls with tumors had single nodules except for 3, each of which had 2 nodules. The overall average of .66 nodule per mouse in the exposed group is significantly greater than the overall average of .31 nodule in the controls; $t = 3.06$; $P < .01$.

Other neoplasms observed in the exposed animals included 3 lymphocytic leukemias. Two of these occurred in males 4 months after the exposure and one in a male 10 months after the exposure. No leukemias were observed in the controls, but since leukemia occasionally occurs in strain A mice, one cannot be certain that these 3 were induced. One experimental female had a hemangioendothelioma of the external ear at 11 months after the exposure. The only tumor other than pulmonary tumors found in the control mice was a hepatoma occurring in a female 11 months after the beginning of the experiment.

One male and 4 females of the experimental group had primary amyloidosis and the resulting nephritis that have been shown to be inherited in Strain A and probably due to a recessive gene(8,9), but usually occurring at a later age. The male also showed extensive granulocytopoiesis in the liver. Six control males and 3 control females had amyloidosis and nephritis but some of these also had dermatitis and the amyloidosis may, therefore, have been secondary.

Discussion. It is impossible to make an accurate comparison between the carcinogenic potency of mustard gas as indicated in this experiment with that of other carcinogens as shown in other tests. In Hartwell's(10) survey of compounds which have been tested, it is noted that few of the well-known carcinogens have been tested by inhalation. Campbell(11) reported somewhat higher incidences of pulmonary tumors in mice that had inhaled chimney soot, exhaust soot, cigarette smoke or road dust than was observed in control mice. There was no indication, however, that genetically homogeneous mice were used, and, thus, it is impossible to estimate the significance of the differences observed. Seelig and Benignus(12) observed an 8% incidence of pulmonary tumors in mice of the old Buffalo strain that had been subjected to an atmosphere of soot dust as contrasted with an incidence of 2% in the control mice. Later (13) they obtained no pulmonary tumors in genetically very resistant mice of strain C57BL that inhaled a 10% mixture of carcinogenic tar in lampblack. Rajewsky *et al.* (14) reported 11 pulmonary tumors in 33 white mice that had inhaled radon, but here also failure to use an inbred strain with a known incidence of pulmonary tumors precludes an estimate of the significance of their results or a comparison between them and those reported herein.

Pulmonary tumor response to many carcinogens, including sulfur mustard, when injected in strain A mice have generally been much greater than that reported here. This, however, is probably due to difference in dosage. While in this experiment it was not possible to estimate the dosage accurately, in view of the fact that only .01 cc of the sulfur mustard was placed in the 8-liter desiccator and that not all of it evaporated, and since the mice were in the desiccator for only 15 minutes, it must be assumed that the amount of sulfur mustard that came into contact with the lungs of each animal was extremely small.

The pulmonary tumor response to mustard gas in this experiment approximates that reported by Lorenz *et al.*(15) in strain A mice that received 2500 r gamma irradiation given at the rate of 8.8 r per 8 hours per day.

Summary. Strain A mice exposed for 15 minutes to vapors from .01 cc sulfur mustard in an 8-liter desiccator had a significantly

higher incidence of pulmonary tumors with a significantly higher average number of nodules than did their littermates kept as controls.

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Age Changes in the Fine Structure of Anterior Pituitary of the Mouse.* (20147)

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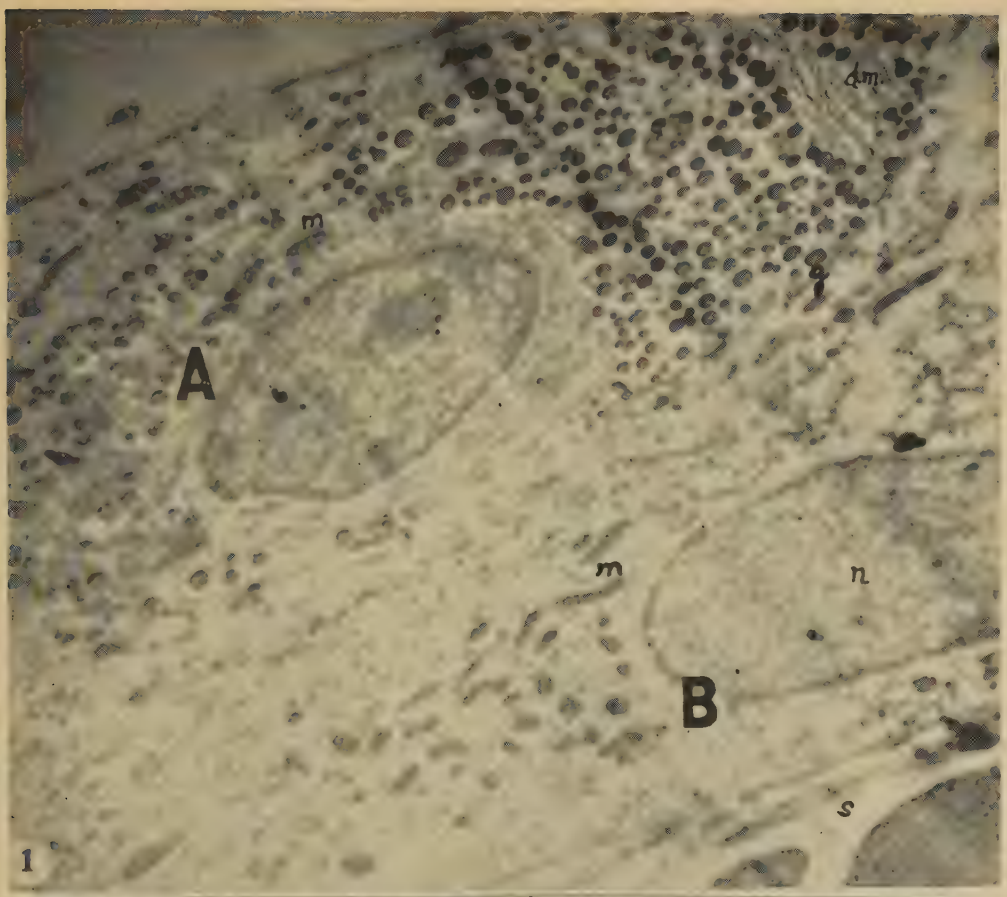
Morphologic changes which have been described in the cells of aging animals include loss of regularity of nuclear and cellular outline(1-3); increased occurrence of cells with giant nuclei(4) and multiple nuclei(5,6); increased amounts of cytoplasmic pigments (1,7,8), and vacuolization of the cytoplasm (3). Recently Payne(9-11) has made extensive observations on cellular changes in the anterior pituitary, adrenal, and thyroid glands of the aging cock. His most striking findings involved swelling and vacuolization of the mitochondria, in some cases so extensive that they destroyed the cell. Jayne(8) has corroborated this work in the adrenal and pituitary of the rat.

To amplify the studies of Payne and others on the morphological changes in cell structure with age, a study of the anterior pituitary gland of the Swiss Albino mouse has been made, using the greater resolving power now available with the electron microscope. In addition to swelling and disruption of the mitochondria, irregularity of the nuclear outline, a disruption of the fine structure of the endoplasmic reticulum, and loss of the highly organized appearance of the young cell have been observed.

Materials and methods. Five Swiss albino mice 1-2 months of age were compared to 6 old individuals ranging in age from 12 to 14 months. The animals were killed by beheading and bleeding, and the pituitaries were dissected out within 5 minutes of the time of death. The procedures used for fixation and embedding closely followed those which had been outlined by Palade(12). The gland was fixed for 4 hours in 1% osmic acid in Ringer's solution buffered with acetate veronal to a pH of 7.5. The material was then washed 3 times in distilled water and dehydrated by successive passage through 50%, 70%, 95%, and absolute ethanol. The tissue was transferred from absolute ethanol to a half and half mixture of absolute ethanol and methacrylate, where it remained for an hour. The methacrylate solution was made up of three parts butyl methacrylate to one part methyl methacrylate. After three washings in 100% methacrylate solution, the tissue was trans-

* This investigation was supported by a research grant from the National Heart Institute, of the National Institutes of Health, Public Health Service.

† Damon Runyon Memorial Fund Fellow.



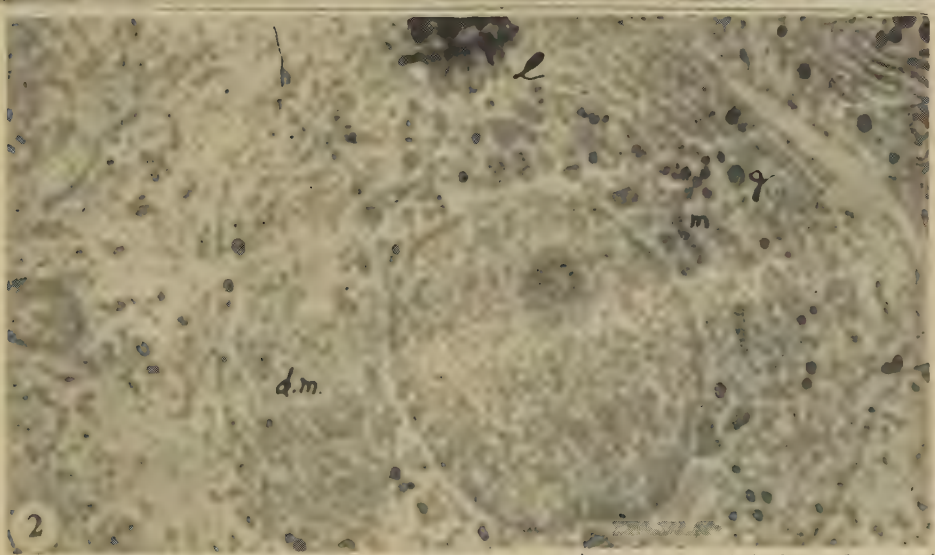


FIG. 1. Electron micrograph of acidophil (A) and chromophobe (B) from the anterior pituitary of a one-month-old mouse. Magnification approximately 6000 ×. l = lipid inclusion, m = mitochondrion, g = granule, d.m. = double membraned system, n = nucleus.

FIG. 2. Electron micrograph of basophil from anterior pituitary of a one-month-old mouse. Magnification approximately 6000 ×.

ferred to methacrylate to which the catalyst, benzoyl benzoate, was added. Polymerization was begun at room temperature for 12 hours, followed by incubation at 47°C for 24 hours. This slow polymerization was found to decrease the incidence of tissue explosion. Sections were obtained by using the Minot microtome, modified as described by Dempsey and Lansing(13), and equipped with a glass knife. Sections were examined without removing the plastic in an RCA model EMU 2a electron microscope with a 40 μ aperture in the objective lens and a centerable condenser aperture. The electron micrographs were taken at an original magnification of from one-to five-thousand times, and were enlarged photographically thereafter as desired.

Observations. Individual anterior pituitary cells as seen with the electron microscope contain a centrally placed ovoid nucleus. The nuclei are bounded by a membrane which in many sections appears to be doubled. The nucleoplasm is granular. Clumps of randomly scattered dense material give the impression of chromatin. A circumscribed dense nucleolus is sometimes seen. Embedded within the finely granular cytoplasm are three classes of formed elements: granules, mitochondria, and a double-membraned system, which Porter(15) and Palade(12) have referred to as the endoplasmic reticulum. The granules are circular in cross-section, homogeneous, and vary considerably in density. They vary in diameter from 0.1 μ to approximately 0.5 μ. The distribution of granules throughout the cytoplasm in random, although there is a suggestion in some of the micrographs that in the neighborhood of capillaries or sinusoids the population of granules is greatest on that side of the cell nearest the circulation.

In the young cells the mitochondria are much as described by Palade(14), being bounded by an outer membrane from which fine double-membraned septa extend inward (Fig. 3). The matrix is homogeneous and of varying density. The mitochondria range in diameter from approximately 0.2 μ to 0.5 μ, and often reach 2 μ to 3 μ in length. They appear in sections as round, ovoid or elongated bodies (Fig. 1 and 2).

The third formed element within the cytoplasm is the double-membraned system which has been described by Porter(15), Palade (12), and Bernhard(16). This system, in section, consists of a double membrane of varying length usually connected at one end and sometimes at both (Fig. 3). It occurs at either an extreme pole of the cell or as a perinuclear cap or demilune (Fig. 1 and 2). Its long axis is usually parallel to the surface of the nucleus. The distance between the 2 components of the double-membraned system varies considerably along its length. At some points the membranes appear to be in contact whereas at other points they may be separated by as much as 0.4 μ.

In both young and old anterior pituitary glands there seem to be 3 general classes of cells. The first group contains cells the cytoplasm of which is almost devoid of the previously described granules and double-membraned system. These we consider to be the chromophobes (Fig. 1). In the second group are cells whose cytoplasm is packed with granules; the double-membraned system, although present, is not a conspicuous element. On the basis of the high content of relatively large and uniform granules we assume that these cells are the acidophiles (Fig. 1). Finally, there are cells which typically contain few granules and large amounts of the double-membraned system. Because of the sparsity of granules and the large amount of endoplasmic reticulum it appears that these cells are the basophiles (Fig. 2). The granules, where present in all 3 groups, do not differ in appearance, nor does the double-membraned system differ from cell to cell. The number of mitochondria is about the same in cells of each class.

The organelles of the cell which show morphologic changes with advanced age are the nucleus, the mitochondria and the double-membraned system (Fig. 4). The changes were present in all cell types. The nucleus becomes irregular in outline, and the nuclear cortex is made conspicuous by a ring of electron dense material. In some specimens the nuclear membrane is recognizable as a fine double membrane, similar to that seen in the young cell.

The most striking age change is found in

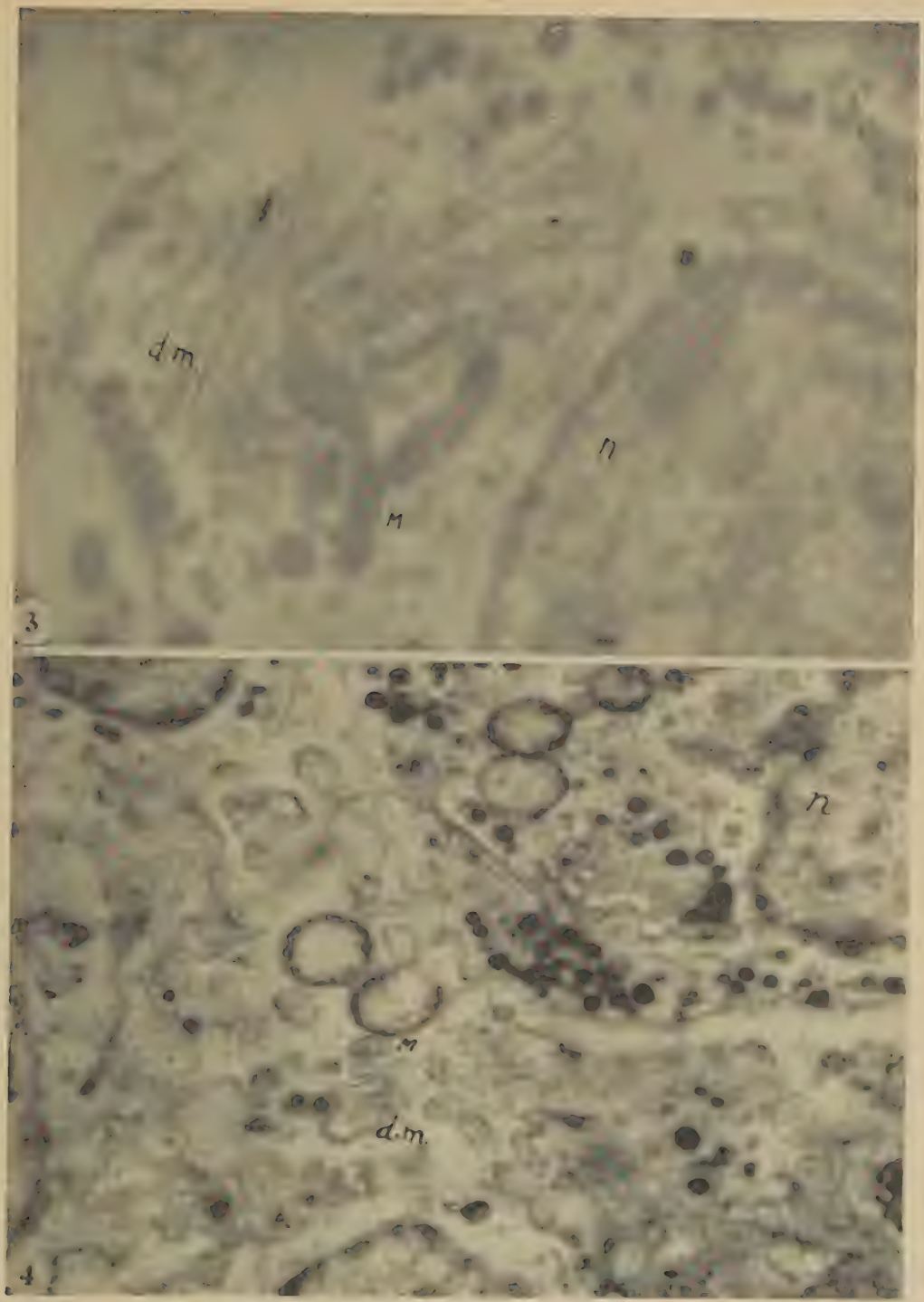


FIG. 3. Electron micrograph at approximately 16000 × of one-month-old mouse anterior pituitary illustrating the architecture of the double-membraned system, mitochondria, and nucleus.

FIG. 4. One-year-old mouse anterior pituitary at approximately 8000× illustrating swelling of mitochondria, disruption of the double membraned system, and increased density of the nuclear cortex.

the mitochondria. Most of the mitochondria in old cells have a diameter from 2 to 5 times greater than those of young cells. They appear to be vacuolated, in that the mitochondrial matrix is very much reduced in amount or changed in kind as evidenced by low electron density. The double-membraned septa, which in youth extend into the medulla of the mitochondrion are reduced to small, inward-directed, stumps. The peripheral membrane is still finely delineated, and at times appears double. Occasional normal mitochondria can be found, as well as numerous intergrades to the most swollen form.

In young cells without exception the double-membraned system is highly organized as a perinuclear cap or as a condensation at one pole of the cell. This orderly localization is completely lacking in old cells, in which the double-membraned system may be found scattered throughout the cytoplasm in a more or less fragmented form. The membranes that make up the system are not well defined and are difficult to resolve.

The effect of the irregularity of the nuclear outline, the swelling of the mitochondria, and the disruption of the double-membraned system is to produce cells which lack the orderly cytoplasmic architecture of the young cell.

Discussion. Payne's cytological analysis of the age changes in the mitochondria of the anterior pituitary, adrenal, and thyroid cells of the cock indicate that there is a progressive breakdown in the structure of mitochondria, involving a vacuolization and accumulation of pigment in these structures. Definite cytological identification of mitochondria depends upon the *in vivo* uptake of janus green by these organelles. Tinctorial reactions such as the Altman(17) and the Bensley-Cowdry(18) procedures, although effective stains for mitochondria, lack specificity. Under such circumstances one might question the identity of structures presumed to be mitochondria which have undergone extensive structural changes. Indeed, in depending upon tinctorial reactions one might fail to recognize mitochondria which either do not stain in the conventional manner or else are too small to be readily visualized in the light microscope.

The description by Palade(14) of a definitive internal structure in mitochondria makes possible recognition of these structures through the use of the electron microscope. We have observed that the mitochondria in the anterior lobe of the mouse pituitary gland conform closely to the description of this organelle by Palade. In addition we found a sizable variation in the diameter of mitochondria including some that were as small as one-tenth of a micron in cross-sectional diameter. Structures of this size probably would not be recognizable in the light microscope.

In the cells of the anterior pituitary gland of old mice the mitochondria are very much enlarged and appear vacuolated. The mitochondrial matrix material is reduced and the double membraned internal septa are compressed against the outer mitochondrial membrane. Despite these marked changes, enough of the internal structure remains in these mitochondria to permit their identification. Although Payne's description of age changes in the mitochondria of the cock anterior pituitary gland emphasized their occurrence in the basophiles, our electron micrographs indicate that these changes appear in all of the 3 cell types.

While structural observations with the electron microscope do not justify functional interpretation, it is reasonable to assume that mitochondria, profoundly altered as they are in aging pituitaries, do not function in the same manner or as effectively as do young mitochondria. There is abundant evidence from many sources that respiratory enzymes (19-21), enzymes associated with the Krebs' cycle(22,23), and enzymes associated with the oxidation of fatty acids(24,25) are at least in part contained within the mitochondria (see also the reviews of A. I. Dounce(26), and Blaschko and Jacobson(27).) There is also evidence that these enzyme systems are dependent upon proper spatial orientation for their function. In their recent review Blaschko and Jacobson state that the enzyme's "spatial arrangement in the cell must clearly be of great importance when the catalytic system is complex." They state that "attempts to separate the components of the dehydrogenase-cytochrome system (apart from the soluble component c) have been unsuccessful. The activity of the preparation

from heart muscle seems to depend on the way in which the single components are built into the framework of the insoluble tissue proteins."

It is quite possible that the architectural disruption of mitochondria in old pituitaries may result in impairment of their enzymatic activities and, in particular, in the respiratory activities of the cells involved. In this connection it should be recalled that various investigators have noted alteration in cellular respiration in certain aged organs(28-30). It would be particularly desirable to determine simultaneously the anatomical and enzymatic changes with age in a single organ.

We have noted that there is a consistent increase with age in the electron density of the cortex of the nucleus. It has not been feasible to determine the source of this added density; it may be that there is a shift in the distribution of chromatin towards the periphery of the nucleus, or that there is an accumulation of insoluble materials in this region. In this connection it may be recalled that calcium increases with age in both the cortex of the nucleus and of the cytoplasm(31). There does not seem to be a significant age change in the fine structure of either the nuclear membrane or cell membrane.

In agreement with the observations of others we have found that the double-membraned system of normal cells is a highly oriented structural complex. According to Bernhard *et al.*(16) the system as seen in the electron microscope is identical with the basophilic component of cytoplasm or the chromidial substance. The presumption is, then, that ribonucleoprotein is concentrated in the double-membraned system. We have recently determined (to be published separately) that exposure of pancreas to ribonuclease results in digestion of this system, a fact which confirms the expectation that ribonucleic acid is a significant component of this organelle.

In the aged pituitaries the double-membraned system is no longer geometrically oriented, and indeed is markedly fragmented. One might expect that in such cells protein synthetic processes may not be carried out as effectively as in normal young cells.

Summary. Electron micrographs of the anterior pituitary gland of the Swiss albino mouse reveal that the cytoplasm of chromophobes, acidophiles and basophiles contain granules, a double-membraned system and mitochondria in varying amounts. The nuclear membrane appears to be double. With aging the nuclear membrane becomes irregular in outline, the nuclear cortex becomes dense, the mitochondria become enlarged and vacuolated, and the double-membraned system fragments. The implications of these observations are discussed.

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Influence of Pregnancy upon Hypertension Induced in Rats by Sodium Chloride and Desoxycorticosterone.* (20148)

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Pregnancy in rats invariably causes a fall of blood pressure when hypertension has been previously induced by a partial constriction of the renal artery(1-3). This reduction of hypertension is manifest in the second half of pregnancy and is maximal just prior to delivery, following which the hypertension returns to the prepregnancy level. The same phenomenon has been observed in dogs(4-6) and in rabbits(2,7). We are not aware of any reports regarding the influence which pregnancy might have upon other forms of experimental hypertension in the rat. Masson, Lewis, Corcoran, and Page(8), however, have stated that the administration of desoxycorticosterone acetate (DCA) to pregnant rabbits results in paralysis or convulsions and hepatic necrosis with subcapsular hemorrhages. Inasmuch as several authors have implicated adrenal cortical-like hormones and sodium chloride in the etiology of human eclampsia (reviewed by Mastboom(9)), the interaction of pregnancy and sodium chloride-DCA hypertension in the rat was studied. Although experimental results in rats are not directly applicable to women, especially because of the recognized differences in placental anatomy and function and the absence of spontaneous toxemia in lower mammals, it was hoped that this study might determine whether there are differences between renal hypertension and sodium hypertension, or whether a syndrome resembling eclampsia might develop in "sodium sensitized" animals.

Methods. Adult female rats of the Long-Evans strain were unilaterally nephrectomized, and 60 or 70 mg of DCA in the form of discs or cylinders were implanted subcutaneously, in the manner described by Masson, Corcoran, and Page(10).† The animals were placed on a diet consisting of 3 parts of Purina fox chow and one part of casein supplemented with the known essential vitamins. One per cent sodium chloride solution was supplied as drinking water with special traps to catch any spill. Systolic blood pressures were determined 2 or 3 times a week, using the microphonic method described by Friedman and Freed(11). Fluid intake and urinary output, quantitative proteinuria and body weights were determined frequently. Since the estrus cycles became quite irregular under this regime, males were placed with the females overnight at arbitrary intervals rather than selecting the time on the basis of estrus. Examinations for sperm

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† We are indebted to Dr. Ernst Oppenheimer of Ciba, Inc. for a liberal supply of DCA cylinders, 15 mg each, and for DCA powder, from which Dr. G. M. C. Masson kindly prepared discs of 35 mg each.

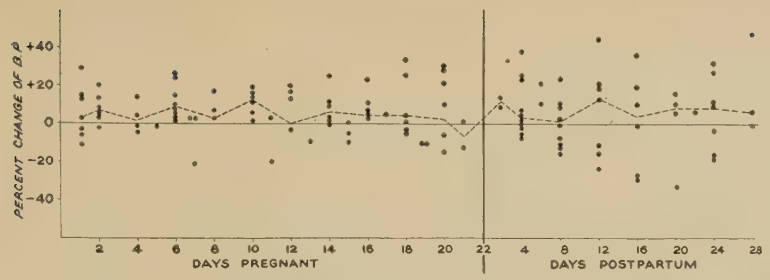

FIG. 1. Percentage change in systolic blood pressure during pregnancy and the puerperium. The zero line represents the mean pressure during the month prior to conception. The dotted line connects the median values.

plugs were made in the morning. After many weeks of failure to find sperm plugs, it was found necessary to substitute tap water for the saline solution during the night of mating. If this were not done, the males became so preoccupied with drinking the salt water that they had either a lack of time or interest in the females.

Results. In view of the fact that Burdick and Konanz(12) reported that DCA administered from the time of copulation prevented implantation of fertilized ova in mice, it is of interest to note the pregnancy success rate in the rats. Of 34 females repeatedly placed with males, 14 delivered live litters successfully. Of the remaining 20, only 2 showed definite sperm plugs without evidence of pregnancy. One additional rat became pregnant but died on the 11th day of a ruptured aneurysm of the duodenal artery. Only a few of the mothers attempted to care for their litters, and of these only a portion of 2 litters survived for more than 10 days. This suggests a deficiency of lactation, but the mothers were not sacrificed for histologic examination of the breasts.

Blood pressure readings during successful pregnancies in 12 animals are shown in the scattergram (Fig. 1) as a percentage change over the prepregnancy level. Of these, 7 rats were hypertensive at the time of conception (systolic pressures between 150 and 170 mm Hg) and 5 had pressures only slightly elevated (112 to 126 mm Hg, as compared with the blood pressures of normal control pregnant rats which average 105 $\pm$ S.D. 6 mm Hg). The dotted line connecting the median values for each 2-day interval during pregnancy and the puerperium indicates that pregnancy exerted no influence whatever upon the level of blood pressure. The fact that the median line is slightly (from 2 to 14%) above the prepregnancy blood pressure level can be attributed to the progressive nature of the induced hypertension.

Fig. 2 portrays graphically the changes in urine volume and urinary protein excretion, using the averages for the entire group of animals. The differences in urine volumes from one period to another are not statistically significant. The increases of urinary protein excretion during the second and third trimesters of pregnancy are statistically significant when compared to the antepartum excretion, and the rates of excretion after delivery are significantly higher than the prepregnancy rates.

Since we did not know whether urine volumes or protein excretion changed during normal pregnancy in rats, 12 untreated animals on stock diets and tap water were observed before, during and after pregnancies. The urinary output varied from 11 to 31

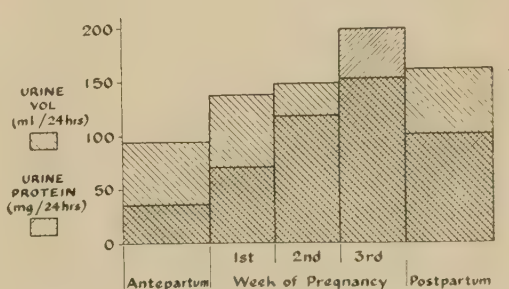

FIG. 2. Mean changes in urine volume and proteinuria during pregnancy and the puerperium in the experimental group.

ml/24 hours, the urinary protein excretion from 4 to 7 mg/24 hours. None of the changes in the group averages was significant.

Observations of body weight and clinical behavior during pregnancy failed to show any evidences of increased edema, muscular irritability or other disturbance in the experimental group as compared to the control group.

Discussion. The administration of DCA and sodium chloride to rats causes hypertension, proteinuria, and a very high fluid intake and output. Superimposing the state of pregnancy causes a significant increase of proteinuria in the latter part of gestation, but no change in the blood pressure or fluid balance. The DCA, in dosages large enough to cause hypertension, does not prevent conception, implantation or normal delivery in the rat.

There are many claims that DCA-like hormones play a role in the causation of human eclampsia, and the present experiments shed no light on this question because of species differences. Suffice it to say that a syndrome resembling toxemia cannot be induced by pregnancy in the rat under the conditions employed. If the degree of proteinuria parallels the extent of renal damage in DCA-treated rats, then pregnancy may accelerate this change. The residual increase of proteinuria during the month postpartum, however, may reflect the natural progression of DCA-sodium disease, since a steady increase of renal damage and protein excretion is characteristic of this syndrome.

One of the chief points of interest centers about the differences between the effect of pregnancy upon this disease and that of induced renal hypertension. We concluded previously that the remission of renal hypertension brought about by late pregnancy could not be duplicated by exogenously administered hormones separately or variously combined (13). We also demonstrated that the lowering of blood pressure was due entirely to the placenta and not the fetus(14). The conclusion was reached that the placenta must exert this effect in one of 2 ways; through a "vascular shunt" effect, or through the liberation of an enzyme (such as angiotonase) which neutralized a renal pressor substance.

The vascular shunt effect is common to both groups of experiments; so one might deduce that the only difference resides in the nature of the chemical mediators of the hypertension. The complete absence of a fall of blood pressure late in pregnancy in the DCA-sodium syndrome, therefore, indirectly suggests that the cause of this hypertension is not the same as the cause of experimental renal hypertension.

Conclusions. 1. Desoxycorticosterone acetate (DCA) and sodium chloride, in amounts sufficient to cause hypertension do not interfere with conception, implantation or normal delivery in rats. 2. When pregnancy is superimposed upon DCA-sodium hypertension, the blood pressure and fluid balance are not affected. There may be a transient increase of proteinuria during the last third of pregnancy. 3. The failure of the elevated blood pressure to fall during late pregnancy, as it regularly does in experimental renal hypertension, suggests a difference in the chemical mediation of the 2 types of hypertension.

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Culture of Mononuclear Cells from Rat Peritoneal Exudate. (20149)

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The need for a convenient source of macrophages in opsonic studies on chicken malaria led to the development of a technic for growing such cells from chicken blood agranulocytes(1). In attempting to adapt this technic to studies of rodent malaria we found it technically impossible to obtain enough rodent blood to provide agranulocytes in the required quantities, and have therefore developed an alternative method for obtaining similar agranulocytes for culture from peritoneal exudates sterilely induced in rats.

The method is here briefly described since it may be of some value to other workers who require cultures of mammalian macrophages or of fibroblasts developing from them, spread in a single layer, which may be exposed to known concentrations of drug, antigen, antibody, etc. in a supernatant phase. Such cultures can be fixed and stained as whole mount preparations, and require no sectioning as do many types of culture employing tissue fragments. Thus the entire contents of each cell rather than sections of a cell are available for study. The described method is further preferable to those using tissue fragments in that contact of the constituents of the liquid phase with each cell is ensured, whereas such contact in the interior of a tissue fragment is at best open to question. *Peritoneal exudate* induced in adult white rats by the intraperitoneal introduction of sterile paraffin oil according to the method of Bloom *et al.*(2) was collected as follows. Two days after injection of the paraffin oil, an anesthetised experimental rat received an intraperitoneal inoculation of 5 to 6 ml of Tyrode's solution modified by omitting $CaCl_2$ to avoid blood clotting, and also containing 1:50,000 Connaught heparin. Following brisk abdominal massage, the abdominal muscles were cut along the midline with aseptic precautions, and the contained fluid, consisting of a milky suspension of cells and paraffin oil in the modified Tyrode's solution, was sterilely collected into a centrifuge tube. Following centrifugation for 5 minutes at 2,000 r.p.m., the plug of semi-solidified paraffin oil was pushed aside, and the cell sediment was transferred to fresh modified Tyrode's solution. After 3 such washings by means of centrifugation, the pooled cell sediment obtained from 2 rats treated as described above was finally suspended in 10 ml of a medium consisting of the following components: 20% normal rat serum; 7% dilute aqueous chick embryo extr.; 73% Tyrode's sol. without $CaCl_2$; 5 units crystalline penicillin g/ml medium; 0.1 ml 0.1% phenol red/ml medium. The *cell sediment* consisted of large mononuclear cells with relatively abundant cytoplasm, as described by Bloom *et al.*

Six 10 mm^2 coverslips were cemented to the floor of each of a series of Carrel flasks with plasma clots. Each flask then received 5 ml of the fluid medium containing the suspension of mononuclear exudate cells. The flasks were closed with rubber stoppers and incubated at 37°C. When cultures were to be maintained for extended periods, the supernatant phase was renewed every 4 or 5 days. The effete supernatant fluid was removed, the cultures were washed for 15 minutes in modified Tyrode's solution at 37°C, and fresh supernatant medium, 5 ml per flask, was added after the washings were discarded. Hypertrophied cells adhered to the floor of the flasks and to the coverslips, and were not disturbed by the rinsing. *Serial observations* on events in each flask were possible, since a single coverslip could be detached and removed without disturbing the rest of the cultures in the flask. In this study cultures were fixed in Zenker-formalin and stained with hematoxylin-eosin-azure stain.

Results. Fourteen-day cultures consisted chiefly of a network of elongate or stellate fibroblasts with long, filiform processes, frequently arranged in a syncytium (Fig. 1). Such cells were often more than 100 μ long, not including the processes, by about 20 to 40 μ wide. Mitotic figures observed in them (Fig. 1) demonstrated that they were actively

FIG. 1. Fibroblast syncytium developing from mononuclear cells of rat peritoneal exudate 12 days after explantation. Note mitotic figure in central fibroblast.

functioning and not merely surviving. Fibroblasts of this type were suitable hosts for obligate intracellular parasites of connective tissue cells, as may be seen in Fig. 2, which depicts colonies of the leishmaniform stage of *Trypanosoma cruzi* developing in such cells 7 days after flagellated crithidial forms had been introduced into the supernatant phase of the culture. Mitoses were still seen in such cultures, even in infected cells, when extensive areas had already been damaged by the parasites. The morphology of the parasites and their relation to the host cells is essentially similar to that described by Hawking(3) and Meyer(4) for cultures of *T. cruzi* in a variety of cell types. Muniz and Freitas (5) grew *T. cruzi* in a medium containing peritoneal exudate, but host cells were not stressed in their study, since their medium supported growth even in the absence of cellular elements.

Numerous macrophages were scattered among the fibroblasts in cultures like those described above. These were generally more or less rounded, about 20 to 40 μ in diameter,

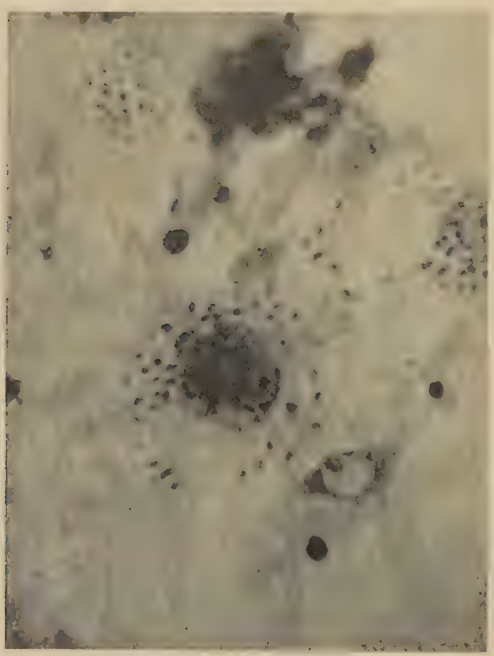

FIG. 2. Leishmaniform stages of *Trypanosoma cruzi* in fibroblasts developing from rat peritoneal exudate 12 days after explantation and 5 days after addition of crithidia of *T. cruzi* to supernatant phase. Parasites in intercellular bridges as well as in the body of infected cell.

FIG. 3. Phagocytosis by a macrophage in a 12-day culture of rat peritoneal exudate, previously exposed to rat blood infected with *Plasmodium berghei*. Several erythrocytes and a number of rod-shaped granules of malarial pigment have been ingested by the macrophage.

with deeply staining, irregularly oval nuclei. Such cells are capable of phagocytosis, as illustrated by the rounded central macrophage in Fig. 3, taken from a culture previously exposed to rat erythrocytes infected with *Plasmodium berghei,* suspended in the liquid phase. The illustrated macrophage contains several erythrocytes in varying stages of digestion, as well as a number of rod-shaped granules of malarial pigment. No phagocytosis was seen in fibroblasts of 2-week cultures in which the macrophages were actively phagocytic.

Summary. A method for culturing a single layer of mononuclear agranulocytes from rat peritoneal exudate is described. Two-week cultures of this type consist chiefly of a network of fibroblasts interspersed with numerous macrophages, and are suitable for the maintenance of obligate intracellular parasites (ex.: *Trypanosoma cruzi,* leishmaniform stage). They may conveniently be used for drug testing, opsonic studies, or other tests requiring the exposure of a single layer of cells, normal or parasitised, to a supernatant liquid phase.

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Influence of Oxygen upon Genetic and Nongenetic Effects of Ionizing Radiation on *Paramecium aurelia.** (20150)

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It has been shown for a variety of organisms that X irradiation under low oxygen tension is less effective in producing biological damage than X irradiation under high oxygen tension. Hollaender, Baker, and Anderson(1) and Giles(2) may be consulted for review. It has been repeatedly suggested that this effect is caused by the reduced quantity of the active materials HO_2 and H_2O_2 produced at low oxygen concentration, implying that a considerable part of the biological effect of X rays must be due to these substances. It is important to find out how far this generalization is true and so to examine carefully any cases in which there is little or no oxygen effect. The present paper reports a series of experiments with *Paramecium aurelia* which show that under certain circumstances oxygen has little or no effect on the production of certain kinds of radiation damage, although under other circumstances it has a marked effect. It seems possible to interpret these findings in terms of changes in the importance of H_2O_2 in producing these effects. Evidence is also presented that oxygen may influence radiation damage in other ways than its effect upon H_2O_2 production.

Materials and methods. Stock 90 of variety 1 of *P. aurelia* was used throughout. The paramecia were selected to be in the first half of the interval between divisions. Shortly after treatment, a number of paramecia, usually 30, were isolated and continued as daily isolation lines.

The technique for measuring the genetic effect at doses of a few kiloroentgens has been described(3). In the present paper, the results are expressed as the percentage of normal exautogamous clones (clones which reach a maximum population in a limited quantity of culture medium in 4 days). This percentage becomes too small to be useful at about 10 kr. However, the percentage of exautogamous clones which survive 4 days, regardless of the

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amount of growth, decreases much more slowly as dose increases and is useful in the range from 10 to about 300 kr.

Nongenetic effects in *Paramecium* were investigated at doses above 30 kr. The two measures used were the number of divisions in the first day expressed as a percentage of the control and the percentage of survivors after one day. These two effects are discussed by Kimball, Geckler, and Gaither(4). In their paper, it is shown that the best general measure of division delay is the reciprocal of the time to the sixth division. With X rays, however, the effects after the first day are so small that, for most purposes, the number of divisions in one day is equally good. Since in some experiments only this measure was available, it has seemed best to use it throughout. In each experiment, unirradiated controls were exposed to high and low oxygen concentrations at the same time and in the same manner as the irradiated animals. There was no evidence from the data that the division rate of unirradiated paramecia was influenced by such relatively brief exposures to different oxygen concentrations. Therefore, the control value for the number of divisions in one day was obtained by averaging the two groups.

The X-ray source was a General Electric Maxitron 250 operated at 250 kvp and 30 ma. For higher doses, including all experiments in which nongenetic effects were investigated, no filters were added. This unfiltered beam is highly heterogeneous and contains a large soft component because of the thin beryllium window of the tube. The measurement of this beam involves difficulties which have not been completely overcome. A nylon Victoreen 250-r chamber was used and measurements were made in air. No attempt was made to correct for scatter and for the difference between the absorption by the walls of the chamber and the exposure containers. The intensity measured in this way was approximately 68 kr/min at 7.7 cm and 16 kr/min at 15.4 cm from the target, which were the two positions used. For some of the investigations of genetic effects at low doses, a 3-mm Al filter was added; and exposures were made at approximately 500 r/min. Other low-dose exposures were made with the unfiltered beam reduced to approximately this intensity by lowering the milliamperage. No significant difference was noted between genetic effects with the 2 types of radiation. High-dose exposures were also made in a Co^{60} γ source kindly made available to us by the Organic Chemistry Section of this laboratory. The source had an intensity of 16 kr/min.

Several different exposure containers and arrangements for achieving the desired oxygen tension were used. For experiments with filtered X rays and with γ rays, 2-ml volumetric flasks, fitted with bubbling tubes, were used. The gas was bubbled through the tubes for 15 min before irradiation, and, in the case of X rays, they were sealed off by stopcocks. Gas was bubbled throughout the exposure in the case of γ rays. The arrangement for X rays was the same as that used by Hollaender and his collaborators in this laboratory for work with microorganisms. Modifications were made so that 2 tubes for different gases could be exposed simultaneously in the limited space within the γ source. Exposure to unfiltered X rays was made in one of 2 kinds of containers. The first was made by cementing, with DeKhotinsky cement, a piece of mica approximately 25 μ thick to a lucite ring 2.5 cm high and 5.5 cm in diameter. This dish was filled with mineral oil to a depth of 1.5 cm, and the paramecia were placed in one or more small drops under the oil on the mica bottom. Irradiation was carried out 7.7 cm from the target with the beam directed upward through the mica bottom. Special boxes were designed which made it possible to bubble with the gas a small volume of fluid containing the paramecia, and to transfer a drop of this fluid to the oil dish and seal off the dish—all under the desired atmosphere. The oil in the dish was also bubbled with the gas. The sealed dishes were removed from the box and irradiated. These same boxes were adapted for exposure of paramecia to H_2O_2 under a desired atmosphere. Small volumes of fluid containing animals and similar volumes of solutions of H_2O_2 were placed in separate, open containers in the box, gas was passed through the box for 20 min, and then the peroxide was added to the animals with a pipette extending through the wall of the box. At the end of 10 minutes, culture fluid con-

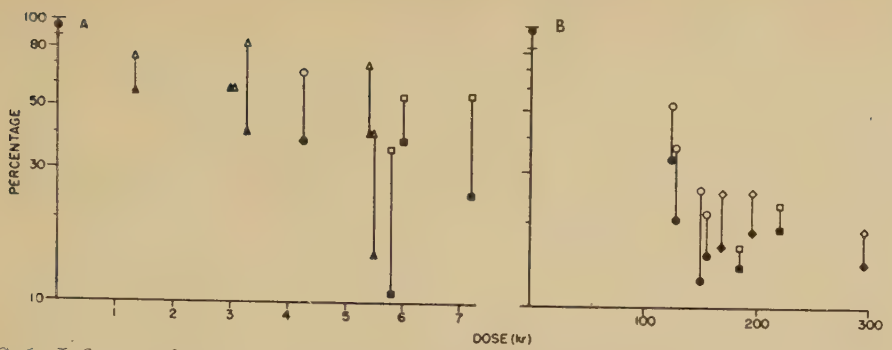

FIG. 1. Influence of oxygen upon genetic effects of low and high doses of X and γ rays. The pairs of points connected by lines represent high- and low-oxygen groups simultaneously given the same dose. Most of the points are average values for 20 irradiated animals with 25 exautogamous clones from each irradiated animal. A. % normal exautogamous clones at low doses. B. % clones surviving 4 days after autogamy at high doses. Open symbols are low oxygen tension; solid symbols, high. Circle = hanging drop. Square = oil dish. Diamond = γ rays. Triangle = 2-ml volumetric flasks and filtered X rays.

taining many living bacteria was added by means of another pipette, thus rapidly destroying the peroxide and ending the exposure. The other exposure dish consisted of a small block of lucite with a well drilled in its center. Two tubes on opposite sides made it possible to pass any desired gas through the well. The paramecia were placed in small hanging drops on a piece of mica 25 μ thick, and this was sealed over the well with hot paraffin. The gas was passed through the chamber for 15 min, the chamber sealed off with stopcocks, and the exposure made. In all cases, the low- and high-oxygen groups for any one dose of radiation were exposed simultaneously.

The gases used were Linde nitrogen (99.9% pure), a commercial mixture of 2% oxygen and 98% helium, a mixture of 20% oxygen and 80% nitrogen, compressed air, and pure (commercial tank) oxygen. In the following, the first 2 will be called low oxygen and the last 3 high oxygen. No consistent differences within these 2 groups have been found.

Genetic effects. The influence of oxygen tension upon the genetic effects of low and of high doses are shown in Fig. 1. In this and other figures, each pair of points representing separate comparisons is connected with a vertical line. Fig. 1 shows that the low-oxygen group is consistently less affected than the high. Exact statements about the magnitude of the effect are not possible but some approximation can be made. Other experiments have shown that both criteria, percentage normal and percentage survival for 4 days, are nearly exponentially related to dose. The curve for the former shows a small upward convexity when plotted on semilog paper whereas that for the latter shows some upward concavity. Thus the ratio of the log of the percentage at high oxygen tension to that at low oxygen tension is a fairly good measure of the oxygen effect. This ratio was obtained for each pair of points separately, and averaged. The average for the low-dose group was 2.6; for the high-dose group, 1.3. This suggests that oxygen has more effect at low dose than at high but does not exclude the possibility that the effect is the same for the 2 groups, since the deviations from a simple exponential relation are such that the value for the low-dose group is an overestimate, the value for the high-dose group, an underestimate.

Nongenetic effects. Fig. 2 shows the data for survival for one day after irradiation. There is reason to believe that in any one experiment the dose curve has a very long shoulder and then drops rapidly from complete survival to complete death(5). The sharp drop, together with variations from experiment to experiment, explains the great scatter of the points. A careful examination of the figure indicates that, at all doses where there was any appreciable difference between the low- and high-oxygen groups, there was more death in the former. This is so for both soft X rays and γ rays. Thus hypoxia increases this effect or does not influence it at

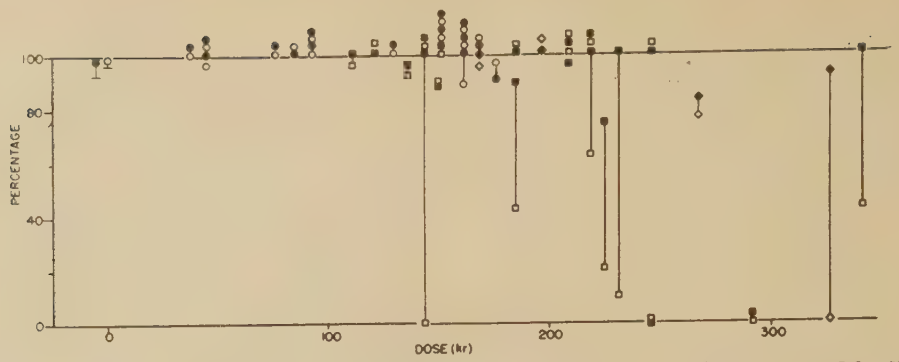

FIG. 2. % survival one day after high doses of X rays. The pairs of points connected by lines represent high- and low-oxygen groups simultaneously given same dose. Most of the points are values for 30 irradiated animals. Average values and ranges are shown for controls. Open symbols are low oxygen tension; solid symbols, high. Circle = hanging drop. Square = oil dish. Diamond = γ rays.

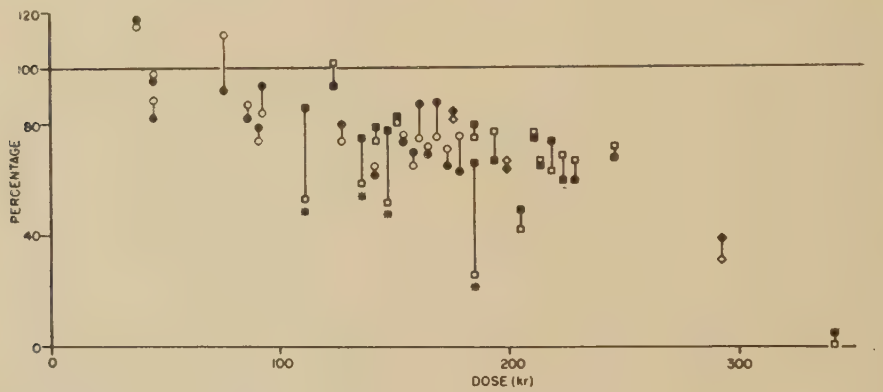

FIG. 3. Divisions in one day among survivors as % of control. The pairs of points connected by lines represent high- and low-oxygen groups simultaneously given the same dose. Many of the points are averages for 30 irradiated animals, but the number was less when survival was low. Open symbols are low oxygen tension; solid symbols, high. Circle = hanging drop. Square = oil dish. Diamond = γ rays.

all. It certainly does not decrease it.

The data in Fig. 3 are for divisions in the first day expressed as a percentage of the control. They agree with data in Fig. 2 in suggesting that, in some cases, hypoxia increases the effect. The main evidence for this comes from the 4 pairs of points marked with asterisks. The high- and low-oxygen groups for each pair are significantly different from one another. However, these 4 points represent all the data from just 2 experiments, one with one dose and one with 3. The rest of the data are consistent with the hypothesis that there was no effect of oxygen; and the whole body of data taken together shows no significant effect. However, the apparent nonrandom distribution of unusual deviations among experiments combined with the evidence in Fig. 2 suggests that the data are really heterogeneous in that a few cases show more effect at low oxygen tension while in the majority the oxygen tension is without influence. In any case, the important point is that there is no evidence that hypoxia decreases the effect.

Irradiation in medium containing few bacteria. The results reported in the foregoing sections were obtained by irradiating paramecia suspended in a culture medium containing many living *Aerobacter aerogenes*. It has been shown(6) that much of the non-genetic damage to paramecia irradiated in a medium containing few bacteria can be attributed to H_2O_2 formed outside the cell. It would be

TABLE I. Effect of Oxygen Tension upon Paramecia Irradiated in Hanging-Drop Preparations in Medium Containing Few Bacteria.

Exp. No.	Dose, kr	Nitrogen N*	Nitrogen S†	Nitrogen D‡	Oxygen N*	Oxygen S†	Oxygen D‡
1	0	0	—	—	30	100	4.1
	92	22	91	2.8	30	0	—
	153	27	0	—	26	0	—
2	0	30	100	4.0	26	96	4.2
	45	30	100	4.0	30	0	—
	91	30	100	2.6	30	0	—
3	0	30	100	3.5	30	100	3.5
	45	30	93	2.7	27	33	0.7
	89	30	83	1.5	30	0	—
4	0	30	100	3.7	30	97	3.1
	38	14	100	3.8	26	54	2.4
	77	29	100	3.0	30	10	1.3

* No. of treated paramecia.
† % survival for one day.
‡ Avg No. of divisions in first day among survivors.

TABLE II. Influence of Oxygen upon Damage by H_2O_2.

Exp. No.	H_2O_2, μg/cc	Nitrogen S*	Nitrogen D†	Air S*	Air D†
1	0	100	4.6	100	4.5
	8	97	3.1	23	2.4
	10	47	1.4	0	—
2	0	100	4.0	100	4.0
	8	50	1.9	30	1.3
	10	23	1.6	20	0.7
3	0	100	3.5	100	4.0
	8	100	3.4	93	2.8
	10	100	3.2	93	2.6
4	0	97	4.3	100	4.3
	8	87	2.3	33	1.6
	10	10	2.3	13	0.8

* % survival for one day, usually out of 30 treated paramecia.
† Avg No. of divisions in first day among the survivors.

expected that paramecia irradiated under the latter circumstances should show a typical oxygen effect on nongenetic changes because of the importance of oxygen in peroxide production. This is shown to be true in Table I. Kimball and Gaither(6) were unable to find any influence of the presence of bacteria upon the genetic effects of X rays.

Oxygen and the action of H_2O_2. The failure to find an intracellular oxygen effect for division delay might, conceivably, result from a balance between a decreased production and an increased effectiveness of H_2O_2 under hypoxia. This hypothesis was tested and ruled out by exposing paramecia to H_2O_2 under different oxygen tensions. The results are shown in Table II. It is clear that instead of an increased effect at low oxygen tension there is a decreased effect. Thus hypoxia should not only decrease the amount of H_2O_2 formed but should decrease its effectiveness. The balanced-action hypothesis is ruled out, unless the improbable assumption is made that H_2O_2 coming from outside the cell differs in this respect from H_2O_2 produced locally. As a further check on this conclusion, samples of H_2O_2 were bubbled with nitrogen and with air for 15 minutes. No difference in concentration could be detected colorimetrically using the starch-iodide method(6).

Discussion. The failure to find an intracellular oxygen effect of the typical kind for division delay and death in one day in *Paramecium* might mean one of 4 things. (1) Insufficient peroxide is produced locally within the cell to be effective. (2) Sufficient peroxide is produced but its formation is not influenced by oxygen tension. (3) The oxygen tension has not been lowered enough in the parts of the cell concerned. (4) Oxygen increases peroxide formation but decreases its effectiveness. The fourth hypothesis has been eliminated by data in the preceding section. The third hypothesis is unlikely in light of the consistent finding of an oxygen effect upon the genetic changes produced by high doses. It is made still more unlikely by the similarity of the results with different methods of removing oxygen. However, it cannot be finally eliminated since localized differences in oxygen concentration are conceivable. The second hypothesis might be true for soft X rays. It is possible that effective concentrations of H_2O_2 are formed only in the dense ionization at the ends of electron tracks and that most of the H_2O_2 in these regions is formed by combination of OH radicals rather than by reactions with oxygen. However, the similarity of the results with γ and soft X rays makes this hypothesis very improbable. Thus the first hypothesis is, by far, the most likely one.

It is made still more likely by the following considerations. We have found that 2 μg/cc of H_2O_2 added to the medium has no detectable effect even after exposures of several hours, whereas a 10-minute exposure to 6 μg/cc produces a distinct delay in division.

Thus there is a limiting concentration below which no effect is produced. Moreover, *Paramecium* possesses an active catalase which would prevent the accumulation of H_2O_2 within the cell, especially if the catalase were in close proximity to the material whose damage leads to division delay and death. Such a location of the catalase is quite possible if the suggestion(6) that division delay and death are the result of changes in the outer portions of the cytoplasm is correct.

Thus we come to the hypothesis that locally produced H_2O_2 is so rapidly destroyed that effective concentrations are not reached within the cell unless it is overwhelmed by large quantities from the outside. Only in this latter case does H_2O_2 become the most important cause of the damage, and only then is a typical oxygen effect found. When peroxide is prevented from accumulating outside the cell, it is even possible that oxygen may act to protect the cell against damage by H atoms by reacting with them to form H_2O_2, against which the cell is well protected. This may explain the greater effect which is sometimes found under low oxygen tension though it does not explain the heterogeneity of the results.

These findings for *Paramecium* agree with those for bacteriophage in suggesting that only when H_2O_2 is involved is the action of X radiation decreased by decreased oxygen tension. Watson(7) distinguishes 3 components to the action of X rays on phage, 1) a direct effect presumably due to energy absorbed within the phage, 2) an indirect effect which he suggests is due to active radicals of short half life, and 3) an after effect due to relatively stable substances. Alper(8,9) makes essentially the same distinction, gives positive evidence that H_2O_2 causes the after effect, and shows that oxygen is necessary for the manifestation of this effect. Hewitt and Read(10) show that the direct effect is not influenced by oxygen, and Alper(9) shows the same lack of effect upon the initial rate of inactivation of phage (presumably Watson's indirect effect) and upon the sensitization of phage to H_2O_2. She believes that this means that oxygen is not needed for the action of radicals during irradiation and is effective only through its role in H_2O_2 formation. The results with *Paramecium* can be considered to support this point of view, at least in showing that oxygen does not enhance the nongenetic effects of X rays except when the conditions are such that these effects are due mostly to peroxide.

The distinct oxygen effect which is found for genetic damage in *Paramecium* could well mean that a considerable portion of this damage is brought about by peroxide. There is no contradiction in the idea that peroxide is effective in the micronucleus but not in the cytoplasm since the catalase content of the micronucleus may be low and only one or a few molecules of peroxide may be sufficient. However, the evidence from *Paramecium* only makes peroxide a possibility and does not exclude other explanations of the oxygen effect.

Finally, we have shown that oxygen may have a 2-fold effect upon the production of radiation damage. It may influence the production of active substances such as peroxide, but it may also influence the biological effectiveness of these substances. Thus, the oxygen effect for paramecia irradiated with few bacteria present consists of at least 2 parts. The presence of oxygen increases the production by irradiation of H_2O_2 in the medium, and it also increases the biological effectiveness of this substance. Similar combined effects may exist in other organisms.

Summary. The nongenetic effects of ionizing radiation upon *Paramecium,* when many bacteria are present during irradiation, are not influenced or perhaps are slightly increased by hypoxia. However, when few bacteria are present, these effects are decreased by hypoxia. The latter finding is in accord with the previously obtained evidence that much of the nongenetic damage produced when few bacteria are present is due to H_2O_2 formed outside the cell. The former finding suggests that H_2O_2 formed inside the cell is not of importance in producing nongenetic damage. On the other hand, genetic damage is consistently less under hypoxia. Previous evidence has shown that this kind of damage is not produced by H_2O_2 formed outside the cell. However, it seems quite possible that H_2O_2 or HO_2 produced locally within the nucleus is of importance in producing genetic damage;

but the evidence from *Paramecium* does not rule out other interpretations; *e.g.*, an effect of oxygen itself upon the recombination of broken chromosomes.

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Adaptation of Type I Poliomyelitis Virus to Mice. (20151)

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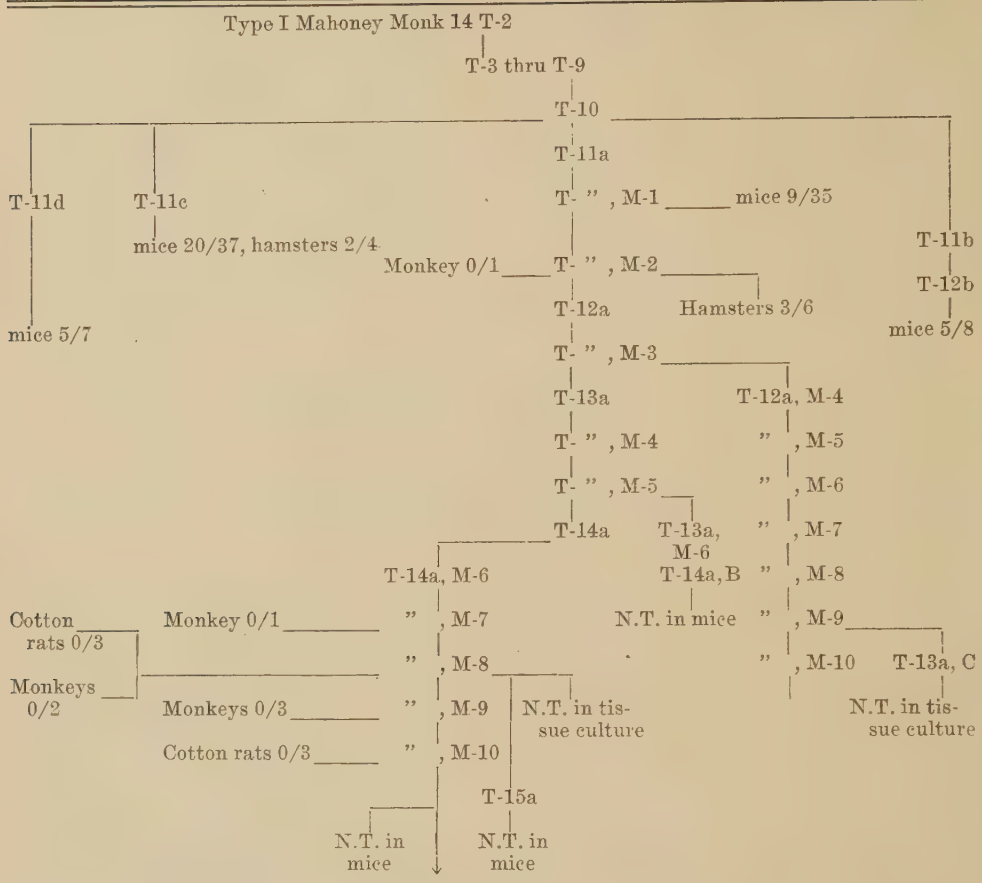
Since the adaptation of the Type II (Lansing) strain of poliomyelitis virus to mice by Armstrong(1), numerous attempts have been made to accomplish this with other poliomyelitis virus strains. Success with the Type III (Leon) strain was reported by Li and Habel (2), employing the intraspinal route in mice (3). This has been confirmed by Casals *et al.*(4), but the low virulence of the adapted strain has limited its usefulness in laboratory and field studies. More recently, Li and Schaeffer(5) have been able to increase the adaptability of the Type III strain to a degree of virulence which enhances its satisfactory utilization in mouse neutralization tests for the detection of antibodies.

Although Steigman and Kokko(6) have shown Type I poliomyelitis virus to persist in the mouse brain, they have not demonstrated that multiplication of the virus takes place. This report will describe methods employed in the successful adaptation of the Type I (Mahoney) strain to mice and its propagation in the rodent host.

Materials and methods. Virus. The Mahoney strain of poliomyelitis was employed in this work. This virus was originally isolated by Dr. Thomas Francis, Jr., from a pool of 3 stools of asymptomatic cases of poliomyelitis in Cleveland in 1941 and was later classified as Type I. The virus, when furnished us by Dr. Jonas E. Salk, had been through a total of 14 monkey passages and 2 monkey testicular tissue culture passages. *Immune serum.* Hyperimmune serum was prepared in monkeys with adjuvants following the procedure of Salk *et al.*(7) against Type I (Brunhilde), Type II (Lansing), and Type III (Leon) monkey propagated stock viruses. These sera were checked for specificity both in mouse and tissue culture neutralization tests. Immune monkey sera prepared and tested against the 3 types of virus in other poliomyelitis laboratories were obtained and also were used in neutralization tests to prove the specificity of the adapted virus. *Animals.* In nearly all the experiments performed, CFW mice were used. These were 3- to 5-week-old mice in the earlier passages, but later 8- to 12-week-old mice were employed because of preliminary indications that the latter may be more susceptible. Hamsters, cotton rats, and C3H mice were tested on 2 occasions. When monkey infectivity tests were conducted, rhesus monkeys weighing 3 to 6 pounds were inoculated intracerebrally. The rodents were successfully infected only by the intraspinal route. Intracerebral passages failed, and combined intraspinal and intracerebral inoculations were not superior. *Tissue culture.* The technic employed in our tissue culture has been described previously(8). Essentially, it

CHART I. Lines of Passage in Tissue Culture and Mice.



T = tissue culture passage; M = mouse passage; N.T. = neutralization test. Fraction = No. paralyzed/No. inoculated animals named.

is similar to methods currently utilized in most laboratories. Fragments of monkey testicular tissue are embedded in plasma clots on test tube walls, immersed in fluid consisting of one part of 5% bovine plasma hydrolysate and 3 parts of Hanks-Simms solution. The tubes are incubated in a slanted stationary position for several days to permit cellular outgrowth. Virus was cultivated in such cultures for 9 to 15 days, after which the tissue fragments in each tube were detached from the tube wall, ground, and suspended in about 0.3 ml of native culture fluid. The suspension was then lightly centrifuged and the supernatant fluid was used for animal inoculation. For serial virus tissue culture passages, only the fluid in amounts of 0.1 ml was transferred to successive tubes. *Neutralization tests.* These were conducted during the course of the work to test the specificity of the virus. Equal portions of heat-inactivated, undiluted immune serum were mixed with virus contained in 10 or 20% mouse cord suspension or in tissue culture fragments ground and suspended in their own fluids. The mixtures were incubated at 37°C for one hour, and 0.15 ml of each was inoculated into sets of tissue culture test tubes in triplicate, or 0.025 ml was inoculated intraspinally into groups of 8 mice for each mixture. Mice were observed for evidence of paralysis for a minimum of 2 weeks and tissue cultures for cytopathogenic changes for 6 days.

Experimental. As mentioned, the Type I virus with which these studies were begun had previously had 14 monkey passages fol-

TABLE I. Direct Passage of Type I Virus in Mice after Second Alternating Tissue Culture Passage.

Passage	Paralysis	Incubation period
T-12a, M-3	5/10*	3^3, 4^1, 9^1†
4	15/19	3^2, 4^2, 5^6, 6^1, 7^1, 8^1, 10^1, 12^1
5	5/13	4^2, 6^2, 16^1
6	4/12	3^1, 4^2, 17^1
7	4/12	4^2, 5^1, 10^1
8	12/19	2^1, 3^2, 4^2, 5^2, 6^2, 7^3
9	7/15	3^2, 5^3, 6^1, 7^1

* No. of mice paralyzed/No. inoculated.
† One mouse paralyzed on 9th day.

TABLE II.
Direct Passage of Type I Virus in Mice after Fourth Alternating Tissue Culture Passage.

Passage	Paralysis	Incubation period
T-14a, M-6	15/19*	3^{10},† 4^4, 7^1
7	16/20	2^1, 3^6, 4^5, 5^2, 7^1, 10^1
8	14/19	2^1, 3^9, 4^4
9	10/20	2^2, 3^4, 4^4
10	12/12	2^2, 3^9, 4^1

* No. of mice paralyzed/No. inoculated.
† Ten mice paralyzed on 3rd day.

lowed by 2 tissue culture passages. In this laboratory, it had 8 additional successive tissue culture passages. Material from these cultures up to the 4th passage failed to produce any apparent infection in mice when inoculated by various routes, including the intraspinal. The remaining 6 passages were made without further tests in mice. From the 10th passage, 4 lines of subpassages were started, each on a different occasion. Series T-11a consisted of alternation between tissue culture and mouse passages with periodic sublines of direct mouse-to-mouse passages. Series T-11b had only 2 additional tissue culture passages, while series T-11c and T-11d had but one. From each of these series, material when inoculated intraspinally into mice or hamsters was found capable of inducing paralytic infection in half, or more than half, of the injected animals.

It became apparent, therefore, that somewhere between the 5th and 11th tissue culture passage a rather striking variation in the behavior of the virus had occurred. This was manifested by its current ability to produce clinical disease in rodents following direct injection into the spinal cord, a characteristic not previously elicited by this technic.

Chart I depicts the lines of passage of the virus in tissue culture and mice, as carried out thus far. The material selected for infectivity and neutralization tests, and the results of some of these tests, are indicated on this chart. In the accompanying tables, there are presented in greater detail selected examples of the more pertinent data obtained in evidence of the selection of a rodent-adapted strain of Type I poliomyelitis virus.

Characteristics of the virus. Mice inoculated intraspinally with sufficient doses of the Type I modified virus develop typical poliomyelitic paralysis. The incubation period is 3 to 5 days, occasionally being as short as 2 days and rarely as long as 10 to 17 days. Paralysis usually appears first in one of the forelegs or hind legs and gradually spreads to all extremities. Some animals may survive in the paralytic condition for several days. In the early passages especially, paralysis was frequently mild and many of the mice recovered.

Virus derived from mouse cord suspensions had a greater infectivity titer for tissue cultures than for mice. In tissue cultures this virus was infective in titers of 10^{-4} to 10^{-5} while in mice infectivity ranged from $10^{-0.5}$ to 10^{-1}. However, when this virus was grown in tissue culture it not only retained the same tissue culture titer but also had a relatively higher (10^{-1} to 10^{-2}) mouse infectivity titer. With increasing numbers of mouse passages, direct or alternate, there appears to be a trend toward increasing virulence for mice (Table II), despite some fluctuation. In the first mouse passages only 9 of 35 mice were paralyzed, while in current passages the virus paralyzes 50 to 100% of the mice. With continued passage of this virus strain, the infectivity titers for mice of both the mouse-maintained and tissue-culture-maintained virus are gradually approaching each other.

The spinal cords of representative mice, occasionally sacrificed and autopsied following infection, when examined microscopically, have invariably revealed the typical histopathologic lesions of poliomyelitis. Small groups of monkeys were inoculated intracerebrally, mostly with late passage virus, but these remained asymptomatic. However, in one monkey, sacrificed 29 days after inocula-

TABLE III. Neutralization Test of Passage Type I Virus in Mice and in Tissue Culture.

N.T. in:	Virus passage	Material	Approx. I.D.	Immune monkey serum (Type) I	II	III	Normal monkey serum
Mice	T-14a, B	Tissue culture undil. susp.	100	0/8	6/8	8/8	7/8
				Cell degeneration in tubes			
				1 2 3	1 2 3	1 2 3	1 2 3
Tissue culture	T-14a, M-8	Mouse cord 20% susp.	10000	— — —	+ + +	+ + +	+ + +

TABLE IV. Specificity Tests on Control Monkey Immune Sera Tested against Heterologous Types of Poliomyelitis Viruses in Tissue Culture and in Mice.

		Virus		Monkey serum immune to: I (Brunhilde)	II (Lansing)	III (Monkey Leon)	Normal monkey serum
Neutralization test in tissue culture				Cellular degeneration in days			
				1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6
	I	Mahoney		— — — — — —	— + + + + +	— + + + + +	— + + + + +
	II	YSK		— — + + + +	— — — — — —	— — + + + +	— — + + + +
	III	Saukett		— + + + + +	— + + + + +	— — — — — —	— + + + + +
Neutralization test in mice							
	I	Mouse Mahoney	Paralysis	0/8	6/8	8/8	7/8
			Incubation (days)	0	3.1	4	4.5
	II	Mouse MEF-1	Paralysis	10/10	2/10	9/10	9/10
			Incubation (days)	2.2	7	4.8	2.2
	III	Mouse Leon	Paralysis	8/9	10/10	0/10	9/10
			Incubation (days)	6	6	0	5

tion, lesions suggestive of poliomyelitis were found in the brain and spinal cord.

Intraspinal inoculation of a group of 4 and a group of 6 Syrian hamsters resulted in paralysis of half of each group, but 2 groups of cotton rats, similarly inoculated with a suspension that paralyzed 10 of 20 mice, have thus far resisted this virus. Intracerebral inoculation has thus far failed to paralyze any of the species of animals used.

There are some interesting differences between the Type I mouse adapted virus and mouse Types II and III. Type III mouse adapted virus does not paralyze hamsters and following serial tissue culture passages loses its virulence for mice. The MEF-1 Type II mouse virus, in adequate doses, infects mice readily by the intracerebral route as well as by the intraspinal and usually paralyzes most or all of the inoculated mice within 48 hours and produces symptoms in cotton rats.

Identity of the virus. At present, the immunologic evidence supporting the identification of this virus as poliomyelitis Type I rests primarily with the results of neutralization tests. These were performed on several occasions during the course of propagation and were conducted in both tissue cultures and mice. Table III illustrates the outcome of a typical test. On each occasion the virus was inhibited by Type I, but not by Types II and III, immune monkey sera prepared and previously tested in this laboratory for specificity (Table IV). In addition, sets of hyperimmune antisera obtained from two other poliomyelitis laboratories were also used to check the specificity of the virus. The results obtained with these sera confirmed the findings shown in Tables III and IV, adding further evidence that the mouse-adapted virus could be correctly classified as Type I. Results of cross immunization and cross infection experiments currently under way are as yet incomplete.

Discussion. In attempting to adapt Type I poliomyelitis virus to mice a number of methods were tested with view toward reducing the resistance of the experimental animals. These

included the use of cortisone, metabolites, exposure to cold, multiple routes of inoculation and other procedures. None of these were successful except the use of modified virus introduced intraspinally. This work illustrates the importance of the intraspinal route in mouse adaptation of poliomyelitis viruses, although the mechanism of pathogenicity by this route is not yet clear. With further passages it is anticipated that a better adaptation of the Type I poliomyelitis virus will be effected as was the case with Type III virus(5). It is especially significant that a virus strain which grows well in extra-neural tissues does not obviously infect when introduced into mice by various routes, including the intracerebral, and yet produces the typical poliomyelitic infection when injected directly into the spinal cord.

It is evident also from the data presented that the route of infection *per se* was not the only factor involved in the successful adaptation of this virus to mice. Obviously some alteration in the properties of the virus occurred in the course of tissue culture passage which, combined with intraspinal inoculation, permitted this adaptation. This may have been a quantitative change, a true mutation, or a temporary variation. It is possible also that this change may be associated with the unmasking of a component which enhances mouse virulence or the elimination of an inhibitor. It may be of significance that in the adaptation, this strain has lost its original virulence for monkeys.

While answers to certain questions must await further investigations which are currently in progress, preliminary tests indicate that this virus strain may be utilized adequately in the performance of relatively rapid neutralization tests in mice and tissue cultures.

This adaptation of the Type I poliomyelitis virus to rodents, when confirmed by other investigators, will provide the long-desired simplified tools with which to conduct, on a more extensive scale, the important field and laboratory studies previously hampered by tedious and expensive methods. Although primary isolation of poliomyelitis virus is not yet possible in the mouse, the adaptation of the 3 virus types to this animal will permit the performance of rapid, reproducible, and relatively inexpensive serologic and immunologic tests.

Summary. 1. The procedures employed in the successful adaptation of Type I (Mahoney) poliomyelitis virus to Swiss mice are described. This virus could also infect hamsters and C3H mice but not cotton rats, while its original virulence for monkeys was lost. 2. The use of the intraspinal route of inoculation and the selection of a mutant or variant of the virus is believed to be responsible for this adaptation. Other possible explanations are discussed. 3. With this attainment, all 3 poliomyelitis virus types have now been adapted to mice. This will permit more rapid progress in field and laboratory studies of poliomyelitis.

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Amino Acid Composition of Thymus Nucleohistone.* (20152)

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The isolation of a nucleohistone from chick erythrocyte nuclei and the microbiological analysis of this protein for its amino acid composition has been described in a previous paper(1). In this communication we wish to report a similar study of nucleohistones derived from bovine thymus gland by a modification of the method of Mirsky and Ris(2) for the isolation of chromosomes.

Experimental.† One hundred grams of fresh calf thymus were minced with scissors and suspended in 450 ml of 0.14 M NaCl. The solution was then homogenized in a Waring Blendor for 7 minutes. A drop of octyl alcohol was added to minimize foaming. The solution was removed from the Blendor and immediately cooled to 0°C, after which the solution was centrifuged for 5 minutes at 1500 G. Following centrifugation, 2 distinct layers of solid material were noted, an upper layer of chromosomal material, and a lower layer of debris and unbroken nuclei. The turbid supernatant liquid contained lighter cellular elements and cytoplasmic material. It was discarded along with the uppermost part of the chromosomal layer. The centrifugate was again suspended in 4 times its volume of isotonic saline and centrifuged for 3 minutes at 1200 G. The clean, white layer of chromosomal material was then separated from the debris with a suction pipet and transferred to a clean test tube where it was resuspended in 4 times its volume of 0.14 M NaCl and washed 4 additional times. The thoroughly washed chromosomal material was then filtered in the cold and approximately 20 ml of 0.2 N HCl were added to extract the histone. The HCl extraction was continued until all of the histone had gone into solution. The acid extract was dialyzed against distilled water overnight, after which 0.05 N NaOH was added to maximum precipitation at pH 10.4. The precipitated nucleohistone was filtered, washed twice with ethanol and once with ether, and finally dried *in vacuo* at room temperature. The dried product was analyzed for total nitrogen by the method of Hiller, Plazin, and Van Slyke(3) and then hydrolyzed in an all-glass reflux condenser. One sample was hydrolyzed with 20% HCl and another, for determination of tryptophan, with 5 N NaOH(4). The hydrolyzates were neutralized and analyzed microbiologically for 18 amino acids by methods previously described(1). Analyses for amino nitrogen were also performed on samples from the hydrolyzates using the method of Van Slyke *et al.*, based on the gasometric measurement of carbon dioxide evolved after reaction of the hydrolyzates with ninhydrin(5).

Results and discussion. The results of the analyses for the total nitrogen and the amino nitrogen of the proteins are shown in Table I. The amino acid analyses on 2 individual samples of bovine thymus nucleohistones, designated T-1 and T-2, are reported in Table II. The serine and threonine figures have been corrected for decomposition of 5 and 10%, respectively, as suggested by Rees(6) who

TABLE I. Nitrogen Analyses of Thymus Nucleohistones, g/100 g.

	Total N.		α-amino N.	
	A.	B.	C.	D.
Sample No.	By Kjeldahl analysis	Calc. from amino acid content (Table II)	By ninhydrin analysis	Calc. from amino acid content (Table II)
T-1	16.35	16.14	11.71	12.64
-2	16.31	16.37	10.42	11.52

* Supported partly by a grant-in-aid from the California Division of the American Cancer Society. The data are from a thesis submitted by Manford D. Morris to the Graduate Division of the University of San Francisco, in partial fulfillment of the requirements for the degree of Master of Science.

† All procedures were carried out at 0°C unless otherwise stated. The centrifuge used was an International refrigerated centrifuge, Model PR-1. When using the Waring Blendor it was placed in the refrigerator at 0°C.

TABLE II. Amino Acid Composition of 2 Samples of Thymus Nucleohistone.

Amino acid	Amino acid per 100 g protein		Contribution of each amino acid to total N. of protein*	
	T-1	T-2	T-1	T-2
	g		%	
Alanine	9.5	10.0	9.5	9.7
Arginine	12.0	12.9	23.5	25.4
Aspartic acid	6.8	6.4	4.4	4.1
Cysteine/cystine	—	—	—	—
Glutamic acid	11.4	11.0	6.6	6.4
Glycine	4.8	4.6	5.5	5.3
Histidine	3.0	3.1	5.0	5.2
Isoleucine	6.6	6.6	4.3	4.3
Leucine	9.6	7.7	6.3	5.0
Lysine	13.2	13.4	15.5	15.8
Methionine	1.0	0.8	0.5	0.4
Phenylalanine	2.4	2.4	1.2	1.3
Proline/OH proline (?)	3.4	4.0	2.5	3.0
Serine	3.2	4.0	2.6	3.3
Threonine	6.0	6.3	4.3	4.5
Tryptophan	0	0	0	0
Tyrosine	4.4	4.1	2.1	1.9
Valine	4.8	4.4	3.5	3.2
Ammonia			1.4	1.6
Totals			98.7	100.4

* These figures were obtained by calculation from the amino acid percentage composition of the protein as shown in the first 2 columns of this table and total N. obtained by Kjeldahl analysis (Table I).

observed this degree of destruction under similar conditions of hydrolysis.

Hamer(7) quantitatively analyzed an acid hydrolyzate of thymus histone by the use of starch columns and accounted for 94.7% of the total nitrogen of the protein by 15 amino acids. The NH_3 nitrogen brought the total nitrogen recovered to 99.5%. Methionine, cystine, and tryptophan were not found in the hydrolyzates assayed on the starch column. Neither cystine nor cysteine was detected by a polarographic analysis but a separate colorimetric determination for tryptophan in the protein indicated a content of 0.04%. This corresponds to about 5 γ of tryptophan per ml of the alkaline hydrolyzates which were assayed microbiologically in our laboratory, a quantity well within the sensitivity of our analytical method even after allowing for some decomposition which should be minimal in the alkaline hydrolyzate. However, in none of our thymus nucleohistones was any tryptophan found. A small quantity of methionine was detected, but cystine was either absent or present in trace quantities which were inadequate for a satisfactory assay. In the study of calf thymus reported by Hamer, a great excess of isoleucine over leucine was found, whereas our analyses indicated that these 2 amino acids were present in about the same amount. The sum of the concentrations of isoleucine and leucine in Hamer's samples is approximately those reported in our study. It is possible that complete separation of the 2 amino acids was not obtained in the chromatographed samples on the starched column. Our findings agree with those of Daly *et al.* (8) in showing essentially equal amounts of leucine and isoleucine in the thymus histone samples.

We have previously noted(1) that the chick red cell nucleohistones were characterized by the presence of an excess of lysine residues over arginine, in contrast to the findings of Hamer, or of Daly *et al.* This is also true of the thymus nucleohistones we have studied. The considerable quantities of alanine reported both in the chick and the thymus nucleohistones isolated in our laboratory also contrast with that reported by Hamer or by Daly *et al.* However, the findings with regard to all other amino acids agree well with those of previous workers. Furthermore, the amino acid constitution of the nucleohistones from the thymus is, in general, quite similar to that found by us for corresponding nuclear protein derived from the chick red cell.

Summary. 1. Two samples of histone isolated from bovine thymus nuclei have been analyzed for their content of 18 amino acids by microbiological methods. Approximately 98% of the total N of the protein was accounted for by 16 amino acids. 2. No tryptophan was found in the thymus nucleohistone samples. 3. The presence of methionine in these proteins was confirmed but that of cystine is questionable. 4. In general, the amino acid composition of the thymus nucleohistone resembles that of the corresponding protein derived from chick erythrocyte previously reported by us.

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Liver Xanthine Oxidase Activity in Relation to Availability of Methionine from Soybean Protein.* (20153)

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It is well known that the nutritive value of the protein from soybean meal can be markedly enhanced by methionine supplementation or by proper heat treatment(1). The demonstration that the amount of sulphur(2) or methionine(3) absorbed from the intestinal tract of the rat is the same whether the animal is fed the raw or heated soybean meal has led to the suggestion that the methionine derived from unheated soybean protein must be absorbed in a form(2), or in a manner(3), which cannot be utilized for growth.

Since the growth depression observed in animals fed raw soybean meal is known to be caused by other factors as well as the availability of methionine(4), it appeared desirable to apply a criterion other than growth as a measure of the utility of methionine from raw and heated soybean protein. The xanthine oxidase activity of the liver was selected for this purpose since the activity of this enzyme has been shown to be a sensitive index of the availability of methionine from dietary protein(5,6).

Experimental. Each kg of basal ration used in this study contained the following components: raw or heated (autoclaved at 15 lb for 20 min.) soybean flour,† 400 g; salts (7), 40 g; Crisco, 60 g; sucrose, 500 g; and vitamin supplements(7) modified to include 40 μg vit. B_{12}. When 0.45% DL-methionine supplemented the basal ration it replaced an equal weight of sucrose. Two types of feeding trials were conducted: *ad libitum* feeding (Exp. A), and paired feeding (Exp. B). In Exp. A, young weanling rats (littermates of the same sex) weighing 35-50 g were distributed into the following groups: (I) raw soy; (II) raw soy + methionine; (III) heated soy; (IV) heated soy + methionine. Each group was composed of 3 females and 5 males; within each group rats of the same sex were housed together in separate cages. Food and water were furnished *ad libitum*, and the weight and food consumption were recorded daily for 6 weeks. The design of Exp. B was identical to that of Exp. A except that each animal was housed in an individual cage, and the food intake of each animal in groups II, III, and IV was restricted to that of its littermate in group I. During the last week of the experiment all animals were transferred to individual metabolism cages for a period of 3 days to permit collection of the feces. The feces were dried in an oven (overnight at 105°C), weighed, ground to a fine powder in a Wiley mill, and 2 g samples were analyzed for methionine using the method of Horn *et al.*(8). Representative samples of each ration were likewise analyzed for methionine. From these data, in conjunction with a measure of the food intake during this period, the amount of methionine absorbed was calculated. At the end of 6 weeks, the animals

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† Nutrisoy XXX, Archer-Midland-Daniels Co., Minneapolis, Minn. According to the manufacturer this product has been extracted with hexane and subjected to a minimum amount of heat treatment.

TABLE I. Growth, Absorption of Methionine, and Liver Xanthine Oxidase Activity of Rats Receiving Diets Containing Raw or Heated Soybean Protein with and without Added Methionine.*

Group	Diet: Soybean flour	Diet: Methionine added	Wt gain† (g/6 wk)	Food intake (g/day)	Methionine absorbed§ (mg/100 g rat)	Xanthine oxidase activity‖: mm^3O_2/hr/g dry wt	Xanthine oxidase activity‖: mm^3O_2/hr/g N
			Exp. A: *Ad libitum* feeding				
I	Raw	.0	69 ± 7.5	6.4‡	69 ± 3.7	332 ± 70	5800 ± 1310
II	"	.45%	120 ± 9.6	8.0	159 ± 4.3	785 ± 95	9700 ± 1500
III	Heated	.0	122 ± 8.7	8.5	63 ± 2.4	650 ± 82	7800 ± 1420
IV	"	.45%	152 ±12.4	9.6	147 ± 4.4	792 ± 84	10200 ± 1630
			Exp. B: Paired feeding				
I	Raw	.0	65 ± 5.3	6.3 ± .4	54 ± 4.6	357 ± 48	5200 ± 580
II	"	.45%	84 ± 7.3	6.3 ± .4	153 ± 8.4	769 ± 65	10800 ± 1810
III	Heated	.0	83 ± 6.0	6.3 ± .4	55 ± 2.6	643 ± 92	8200 ± 1310
IV	"	.45%	86 ± 6.6	6.3 ± .4	154 ± 9.7	787 ±103	11000 ± 1760

* All values are means ± stand. error of the mean based on 8 rats per group.

† Based on the "t" test(15) significant differences between groups are as follows: Exp. A and B—I vs II, III, IV ($P < .01$); Exp. A—IV vs II, III ($P < .01$). All other differences are not considered significant.

‡ Stand. error of the mean could not be calculated since individual food consumption data were not available in the *ad libitum* feeding trials.

§ $\frac{\text{Methionine ingested (mg)—methionine in feces (mg)}}{\text{Body wt (g)}} \times 100$. Based on food intake and fecal excretion covering 72 hr. "Body wt" is the avg wt during this same period.

‖ Based on the "t" test(15) significant differences on either a dry wt or N basis are as follows in both Exp. A and B: I vs II, III, IV ($P < .01$); II vs III ($P < .05$); and III vs IV ($P < .05$). All other differences are not considered significant.

were sacrificed by decapitation, and the livers removed and placed immediately on cracked ice. Homogenates were prepared and the xanthine oxidase activity determined in the manner described by Axelrod and Elvehjem (9). In any one run littermates representing each group were selected to provide a more valid comparison between groups. Aliquots of the homogenates were removed for dry weight and nitrogen (micro-Kjeldahl) determinations. In order to compensate for possible variations in liver composition, enzymic activity was calculated on a nitrogen as well as a dry weight basis.

Results and discussion. The data presented in Table I clearly illustrate the beneficial effect on growth produced by heating the soybean flour or by adding methionine to diets containing the unheated flour, this effect being more marked with *ad libitum* feeding. In the latter instance also, methionine added to the ration containing heated soybean flour elicited a further growth response indicating a suboptimal level of methionine even in the heated soybean protein. The activity of xanthine oxidase in the liver, calculated on either a dry weight or nitrogen basis, was likewise increased as a result of heat treatment or methionine supplementation. Heat treatment, however, was somewhat less effective in this respect than the addition of methionine. These data show further that a difference in the quantity of methionine absorbed can be excluded as a possible cause for the enhancement of growth and xanthine oxidase activity effected by heating the soybean flour.‡ The results of the paired feeding experiment also exclude the possibility that a difference in food consumption is a factor contributing to the low level of xanthine oxidase activity in the livers of rats consuming the unheated soybean meal.

One is therefore led to the conclusion that the methionine derived from unheated soybean protein must be inefficiently utilized not only for growth but also for the maintenance of normal levels of xanthine oxidase in the liver. A closer inspection of the data, however, reveals that another factor in addition to the availability of methionine is affecting

‡ This statement is based on the assumption that the short term experiment involving the measurement of methionine absorption is a true reflection of the entire experimental period of 6 wk.

growth. This is particularly apparent if groups II and IV in Exp. A are compared with respect to growth and enzymic activity. Although the xanthine oxidase values of these 2 groups would indicate little difference in the availability of the absorbed methionine, the growth attained by the rats in group IV was significantly greater. The nutritional superiority of heated soybean flour over the raw flour under conditions wherein methionine is no longer limiting has been attributed in part to the growth-inhibiting effect of "soyin," a toxic protein recently isolated from unheated soybean flour(10,11). Since "soyin" was found to limit growth by causing rats to eat less food(11), it is not unexpected that the restriction of food intake in the presence of added methionine obliterates the difference in growth response observed in *ad libitum* feeding trials (compare groups II and IV in Exp. A and B).

These results further emphasize the necessity for specifying the function of an amino acid when referring to its availability from dietary protein. It had previously been reported that the methionine derived from raw soybean protein was equivalent in lipotropic activity to that originating from the heated protein(12). On the other hand, the data presented here show that the methionine requirements for growth and xanthine oxidase activity are only partially met with the unheated soybean flour as a source of protein. Since changes in xanthine oxidase activity, which accompany modification of the dietary protein, have been shown to involve a synthesis of the enzyme *per se*(13), it appears that both growth and xanthine oxidase activity are functions dependent upon a synthesis of protein. Carroll *et al.*(14) reported evidence which indicates that the bulk of the nitrogen from unheated soybean protein is not absorbed until it reaches the cecum or colon, an effect presumably due to the trypsin inhibitors known to be present in the raw meal. It is possible that the methionine absorbed from the large intestine is rendered ineffective as a participant in protein synthesis without suffering any impairment in its function as a donor of labile methyl groups.

Summary. Xanthine oxidase activity in livers of rats consuming soybean protein was increased by feeding heat-treated soybean flour or by adding methionine to diets containing the unheated flour. Since there was no difference in the degree to which the methionine was absorbed, it was concluded that heating increased the availability of methionine for the synthesis of enzyme protein. The poor growth on raw soybean flour was not entirely due to an impairment in the availability of methionine.

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Passive Immunity in Poliomyelitis VI. Antibody Decline in Rhesus Monkeys Inoculated with Homologous Lansing Antiserum.* (20154)

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We have previously reported that the intramuscular inoculation of monkey immune serum conferred a significant degree of protection on rhesus monkeys challenged intracerebrally with 50 or 100 PD_{50} of Lansing virus(1). These animals were bled at weekly intervals for 6 weeks following the inoculation of serum in order to correlate the outcome of challenge with serum antibody titer, for it appeared probable from the studies of Morgan(2) that a titer of 3.0 would ensure protection. As an additional contribution to the study of passively administered poliomyelitis antibody in monkeys, we now report our findings in a group of 9 rhesus monkeys that survived a cerebral challenge with Lansing virus. The antibody titers have been determined on surviving monkeys in a passive protection experiment previously referred to as Exp. No. 2(1). Bodian(3) has reported that the intramuscular inoculation of human gamma globulin containing poliomyelitis antibodies protected a significant percentage of rhesus monkeys against intramuscular challenge with poliomyelitis strains. In a group of uninfected animals inoculated with gamma globulin, Lansing antibody declined at a constant logarithmic rate.

Materials and methods. Details of passive protection experiment. The details of Exp. No. 2 have already been given(1). To summarize, 20 control rhesus monkeys were inoculated intramuscularly with 5 ml of normal rhesus serum per lb of body weight. A group of 20 monkeys of similar weight received the same dosage of Lansing hyperimmune rhesus serum (titer 3.5 in mice). On the day following the inoculation of serum, all 40 animals were challenged by the thalamic route with 50 PD_{50} of Lansing virus monkey cord pool. Small amounts of blood (5 ml) were taken from all monkeys the day prior to the inoculation of serum and at weekly intervals thereafter, for 6 weeks. Of the 20 monkeys in the control group, one died of bacterial meningitis; 18 of the remaining 19 developed typical prostrating paralysis. The single monkey that escaped paralysis (A409) showed no histologic evidence of poliomyelitis. Of the 20 monkeys inoculated with immune serum, 3 died of bacterial meningitis and one of diarrhea. Only 4 of the remaining 16 developed poliomyelitis; the 12 surviving animals showed no clinical or histologic evidence of poliomyelitis. *Antibody determinations.* Monkey sera were tested for the presence of Lansing virus neutralizing antibody by cerebral inoculation (0.03 ml), in groups of 8-10 mice (12-14 g), of mixtures of 10-fold dilutions of unheated serum and enough Lansing mouse pool virus to give a final 100 LD_{50}. This technic has been employed in these laboratories for several years. The mice were observed for 4 weeks, and the 50% neutralizing endpoints calculated by the Kärber method(4).

Results. Antibody titers in monkeys receiving normal monkey serum. The serum of monkey A409, that failed to develop paralysis when challenged, neutralized Lansing virus (serum titer 1.6); a similar result was obtained when heat-inactivated serum was tested. The sera of all other monkeys were free of Lansing antibody. *Antibody titers in monkeys receiving immune monkey serum.* Four animals in this group died from nonspecific causes and their sera were not tested further. The antibody titers in the 4 monkeys that developed prostrating paralysis, and in 9 of the 12 that remained healthy, are shown in Table I (antibody levels were not determined on the remaining 3 monkeys). All sera obtained before inoculation were free of Lansing antibody. The average titer of sera obtained one week after inoculation in

* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Lansing Antibody Titers in Monkeys in Passive Protection Experiment (No. 2).

Reference No. of monkeys	Result of cerebral challenge (50 PD_{50} of Lansing virus)*	50% serum titers at following periods†					
		Before inj. of serum	Wk after inj. of serum				
			1	2	3	4	6
A423	Prostrating paralysis	0	1.0				
424		0	2.1	1.75			
437		0	2.0	1.6	1.2		
442		0	2.4	1.8			
Avg 50% serum titers:			1.87	1.72			
A425	No evidence of poliomyelitis clinically or histologically	0	2.1	1.9	1.3	1.3	.4
426		0	1.4	1.0	1.0	.9	−.2
427		0	2.2	2.0	1.0	.8	.5
428		0	1.9	1.3	.8	.4	−.1
430		0	1.8	1.0	1.5	.5	.5
431		0	2.2	1.1	.9	1.0	.0
432		0	1.5	1.7	1.2	1.2	.8
433		0	2.2	1.9	1.6	.8	.7
434		0	1.6	1.0	1.1	.6	.0
Avg 50% serum titers:			1.88	1.43	1.16	.83	.29

* Challenge carried out on day following inoculation of 5 ml normal or immune serum/lb body wt.

† Titers expressed as negative logarithms.

the 4 monkeys that became paralyzed was 1.87, and in the 9 monkeys that did not develop paralysis, 1.88. In these 9 monkeys, titers fell to a level that was just detectable 6 weeks after injection of antiserum. The average titers in the 9 monkeys mentioned are plotted in Fig. 1, from which it may be seen that the relationship between the average titers and the time following the injection of antiserum is linear over the period one to 6 weeks, the concentration of antibody falling at a constant logarithmic rate of 10.2% per day. If one assumes that the antibody declined at the same rate during the first week following injection of serum, then the half-life would be 6.8 days, and, by extrapolation, the initial serum titer would be approximately 2.1.

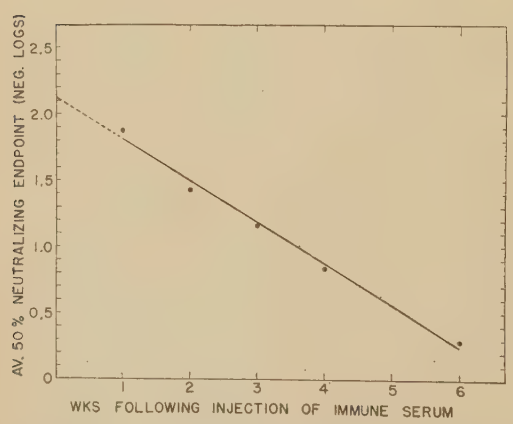

FIG. 1. Rate of decline of Lansing antibody in serum of 9 rhesus monkeys challenged by the cerebral route with Lansing virus in passive protection experiment, and failing to develop evidence of poliomyelitis.

Discussion. No essential difference was found between the average neutralizing titers of sera obtained one week following the injection of antiserum in 9 monkeys that remained healthy following challenge, and in 4 that developed poliomyelitis. An initial antibody titer of approximately 2.1 was sufficient to protect 9 of 13 challenged monkeys. Presumably, therefore, a titer in excess of this figure would protect all monkeys challenged with 50 PD of Lansing virus. The rate of antibody decline in the 9 rhesus monkeys inoculated with Lansing immune rhesus monkey serum, and which survived a cerebral challenge without developing clinical or histologic evidence of poliomyelitis, was almost exactly the same as in a number of normal monkeys receiving human gamma globulin in the experiments of Bodian(3). The half-life of the passively administered antibody was 8.0 days in the study of Bodian and 6.8 days in our experiment. A similar figure (6.6 days) was obtained by Dixon *et al.*(5), who studied the

elimination in rhesus monkeys of homologous gamma globulin labelled with I^{131}.

In the study of Bodian, the initial serum antibody level was found to be approximately 2.0 in monkeys receiving 10 ml of human gamma globulin per kilo body weight. A similar initial serum antibody titer was determined by extrapolation in our experiment in which the monkeys received a corresponding dosage. The Lansing antibody titer of the gamma globulin used by Bodian was 2.8, and the immune monkey serum used in our study had a titer of 3.5. It should, however, be realized that the 2 studies are not precisely comparable, because the monkeys used by Bodian were normal, whereas our monkeys had received an injection of Lansing virus intracerebrally.

Our observations on the fate of Lansing antibody in rhesus monkeys were made as part of a wider program on passive immunity, in which experiments were carried out on humans. In a study previously reported(6), we found that when human gamma globulin containing Lansing antibody was administered to 6 children and 4 adults, whose serum was initially free, antibody could be detected in small amounts up to the 6th week following injection. It is of considerable interest that in the extensive field trials of Hammon *et al.* (7-9), human gamma globulin showed a protective effect against the development of paralytic poliomyelitis for 5 to 6 weeks. These observations suggest that the half-life of poliomyelitis antibody passively injected into humans is considerably longer than the period of 7 to 8 days for the same antibody injected in rhesus monkeys. In the studies of Dixon *et al.*(5), the half-life of human gamma globulin was found to be approximately 13 days for adults, and 20 days for children up to 8 years of age. The half-life of placenta-passing Rh antibodies in newborn infants was found by Weiner(10) to be approximately 30 days.

Summary. 1. Serum antibody titers have been determined in a passive protection experiment in which rhesus monkeys were inoculated intramuscularly with Lansing antiserum prepared in rhesus monkeys; the monkeys were challenged thalamically with 50 PD_{50} of Lansing virus. 2. Four of these monkeys developed paralysis, and 9 remained clinically and histologically normal. 3. Average serum antibody titers in samples removed one week after inoculation were $10^{-1.87}$ in the paralyzed group, and $10^{-1.88}$ in the unaffected group. 4. By extrapolation, the initial antibody titer was estimated to be $10^{-2.1}$. The average titer 6 weeks after inoculation was $10^{-0.29}$. 5. The rate of decline of antibody in the 9 monkeys that survived the cerebral challenge was constant, the half-life being 6.8 days.

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Oral Administration of Co^{60} Vitamin B_{12} in Pernicious Anemia.* (20155)

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Welch *et al.*(1) reported the fecal excretion of 70-95% of radioactive cobalt after an orally administered dose of 0.5 μg of Co^{60} vit. B_{12} in pernicious anemia in remission. The addition of normal gastric juice or concentrated hog stomach reduced the excretion to 5-30%. One subject excreted 70, 95, and 95% activity of 3 administered doses. When 10.0 mg of hog stomach concentrate were ingested, the stools contained only 25% of this activity. One hundred mg of this extract did not affect the amount excreted. Smith(2) demonstrated in the rat that orally administered Co^{60} vit. B_{12} is found in the stool largely in a conjugated or degraded form but not as ionized cobalt(2).

The present study was undertaken to determine the effect of various agents on the excretion of radioactive cobalt after oral administration of Co^{60} vit. B_{12} to 4 patients with pernicious anemia. All were in satisfactory clinical and hematologic remission. One of the persons was receiving 30 μg of vit. B_{12} intramuscularly twice weekly, another 30 μg parenterally every 2 weeks. The 2 remaining cases had not been treated for 6-9 months because of failure to attend the clinic. The subjects were in the lower economic group but apparently consumed adequate nutritional diets. No effort was made to limit the diet in any respect during the period of the experiment. The radioactive vit. B_{12} used in these studies had a specific activity of about 245 μc per mg. A standard was prepared consisting of 1.0 μg diluted in 280 cc of water and set up in a paraffin lined sealed waxed cardboard container. Measurements were made by placing the container directly on a well shielded scintillation counter. The dose administered to the patients (1.0 μg) was adjusted to equal the activity of this standard. It was given in the morning in 280 cc of water. Breakfast was withheld until noon. Each person was instructed to defecate into a waxed container identical in size with the standard. These were identified by name, date, and time of evacuation. Specimens of stools were diluted with water to 280 cc and stirred until a homogenous mixture was obtained. Calculations of excretion were based on a comparison with the known standard. Two to 3 stools were collected and measured for radioactivity after background values were attained. No urine excretion studies were made in pernicious anemia cases. After base line fecal excretions were determined the patients received in addition to 1.0 μg of Co^{60} vit. B_{12} 2.0 mg of folic acid, 15.0 mg of a known potent intrinsic factor prepared from hog duodenal mucosa, 50.0 g of ventriculin,† a "primary period" of 10.0 mg of folic acid orally, daily, for one week prior to administration of the radioactive vit. B_{12}, 150 cc of neutralized normal gastric juice (3 cases) and 2.0 g of aureomycin daily for 6 days (3 cases). Five healthy normal persons and 2 patients in mild cardiac decompensation served as controls. Urine from this latter group was examined for radioactivity.

TABLE I. Radioactivity of Stools Following Oral Administration of 1.0 μg of Co^{60} Vit. B_{12} to 7 Normal Subjects.

Patient	Age	Total fecal excretion of Co^{60}, %	Days to reach max. excretion
H.N.	53	12	5
N.N.	48	21	5
N.A.	30	39	3
N.R.	30	30	3
F.A.	24	36	9
L.M.	47	47	4
S.M.	28	59	3

Results. Summaries of the data are pre-

* Radioactive vit. B_{12} was supplied by Merck and Co., Rahway, N. J. Expenses for these studies were partially defrayed by grants from E. R. Squibb and Sons, New Brunswick, N. J., and Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

† Manufactured by Parke, Davis and Co., Detroit, Mich. (Two different batches used.)

TABLE II. Radioactivity of Stools Following Oral Administration of 1 μg of Co^{60} Vit. B_{12} and Various Agents to 4 Patients with Pernicious Anemia in Remission.

Patient	Age	Co^{60} B_{12}, %	Co^{60} B_{12} + intrinsic factor, %	Co^{60} B_{12} + 50 g ventriculin, %	Co^{60} B_{12} + 2 mg folic acid, %	Co^{60} B_{12} preceded by 1 wk of 10 mg folic acid daily, %	Co^{60} B_{12} + 150 cc gastric juice, %	Co^{60} B_{12} + aureomycin, %
M.S.	50	98	80	90* 91†	99	94	70	96
L.F.	65	93 89	51	75*	55	91	73	93
J.M.	68	99	85	99†	95	96	—	—
L.G.	52	97	98	98†	99	92	67	97

* Ventriculin A. † Ventriculin B.

sented in Tables I and II. It is evident that under the conditions of this experiment the normal subject excreted between 12 and 59% activity of the orally administered dose of Co^{60} vit. B_{12} in 3 to 9 days. Unfortunately, the supply of material did not permit repeated studies in the same subjects to determine if the amount excreted is constant for the individual. Such investigations are now being pursued with a radioactive compound having a lower specific activity. The 2 persons (L.M. and S.M.) showing 47% and 59% excretion, respectively, had adequate free HCl in the gastric juice. Urine specimens from cases H.N. and N.N. failed to disclose any radioactivity for periods up to 8 days after ingestion of vit. B_{12}.

The patients with pernicious anemia showed an excretion of radioactivity varying from 89-99% of the administered dose. Addition of 2 mg of folic acid had no effect in 3 subjects but significantly reduced the excretion about 39% in patient L.F. Oral administration of 10 mg of folic acid for one week prior to ingestion of Co^{60} vit. B_{12} produced no change from the "pernicious anemia pattern" in any of the cases. With the use of a known potent intrinsic factor patient L.F. showed a reduction in excretion of about 45% but no significant change was observed in the other 3 patients. She also had a decrease in excretion when the dose of Co^{60} vit. B_{12} was given with 50 g of ventriculin, whereas the other patients maintained a high excretion rate. The 3 subjects given 150 cc of neutralized normal gastric juice showed a reduction of about 30% in excretion. Aureomycin, 2.0 g daily for one week, had no effect.

In a series as small as the present one it is difficult to reconcile these results with those of Welch and his coworkers(1). The latter administered 0.5 μg of Co^{60} vit. B_{12} (0.03 μc), whereas in the present study each patient received 1.0 μg with a specific activity of 0.245 μc. More patients with pernicious anemia, both in relapse and in remission, are being investigated, using a compound having a specific activity of 0.03 μc/μg.

Conclusions. Following oral administration of 1.0 μg of Co^{60} vit. B_{12}: 1. Seven normal persons excreted 12-59% of radioactive cobalt in the stool in 3-9 days. 2. No activity could be found in the urine of 2 normal subjects up to 8 days after ingestion of the vitamin. 3. Four patients with pernicious anemia in remission excreted 89-99% of the dose in the stool. 4. In 3 of these cases 2 mg of folic acid, 10 mg of folic acid for one week, 50 g of ventriculin, and 15 mg of intrinsic factor had no appreciable effect on the amount excreted. 5. In one case 2 mg of folic acid and 15 mg of intrinsic factor reduced the excretion of Co^{60} about 40%, and 50 g of ventriculin cut the fecal activity about 25%. 6. In 3 patients 2 g of aureomycin for one week had no effect and 150 cc of neutralized normal gastric juice reduced the fecal activity about 30%.

The author expresses his deep gratitude to Dr. E. H. Reisner, Jr. and Dr. Robert F. Schilling who supplied him with Co^{60} vit. B_{12} after the initial supply was exhausted.

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Effect of Vitamin B_{12} on Wound Healing.* (20156)

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Vit. B_{12} has been found to be an important growth factor in young animals(7,8,11) and several investigators believe the vitamin participates in protein synthesis(2,5,13,14). Numerous studies have emphasized the significance of body proteins in wound healing (1,3,9,10,12). It seemed appropriate, therefore, to investigate the role of B_{12} in wound healing of animals with normal and depleted protein stores.

Method. Sherman rats weighing about 300 g were used. They were fed Rockland Chow diet prior to the study and under observation they were given one of 3 diets for 3 weeks before wound infliction and for 6 days post-operatively. A synthetic diet containing 19% protein originally used by Darrow(4) was given to one group of the animals. This was composed of: lactalbumin 18, Crisco 22, dextrin 32, sucrose 25, cod liver oil 1, powdered yeast 2, bone ash 2, NaCl 1, and KH_2PO_4 2 g. The second group received a 0.9% low protein diet containing the same components except that lactalbumin was omitted and the proportions of Crisco, dextrin and sucrose were increased to 26, 36 and 29 g respectively. Both these synthetic diets had approximately the same caloric value of about 500 calories

TABLE I. Weight Changes of Animals on Specified Diets and Medication* before and after Operation.

Diet†	Medication	No. of rats in group	Wt before diet	Wt after diet for 3 wk	Avg daily intake (g)	% wt change on diet	Wt at death	Avg post-operative daily intake	% wt change since operation
Balanced synthetic	Saline for 6 p‡ days	6	313	358	15.6	+14.4	343	15.0	—4.2
"	Saline 21 pr and 16 p days	6	306	344.3	17.7	+12.5	345	16.7	+ .7
Rockland chow	Saline 21 pr and 6 p days	10	307	331	18.6	+ 7.8	321	16.1	—3.0
Balanced synthetic	Vit. B_{12} 6 p days	10	301	339	15.7	+12.3	331	8.9	—2.4
"	Vit. B_{12}, 21 pr and 6 p days	8	279	312	13.5	+11.9	305	9.3	—1.9
Rockland chow	Vit. B_{12} 6 p days	10	322				325		+ .9
Low protein	Saline 6 p days	17	324	279	12.6	—13.8	278	11.5	— .4
"	Saline 21 pr and 6 p days	11	328	275	14.2	—16.4	270	11.6	—1.9
"	Vit. B_{12} 6 p days	14	296	252	10.9	—14.9	241	7.2	—4.0
"	Vit. B_{12} 21 pr and 6 p days	8	303	258	11.3	—14.8	250	8.9	—3.1

* Rats given vit. B_{12} received .5 μ/100 g body wt/day. Daily dosage of isotonic saline to all animals was $\frac{\text{wt of rat}}{200}$.

† All specified diets given 3 wk before operation and 6 days post-operatively.

‡ p = post-operation; pr = pre-operation.

* This work supported in part by a grant from the Medical Research and Development Board, Office of the Surgeon General, Department of the Army.

TABLE II. Data to Determine Effect of Vitamin B_{12} on Tensile Strengths of Wounds in Rats Fed Control and Low Protein Diets.

	Balanced synthetic diet			Rockland chow diet	Low protein diet			Balanced synthetic minus low protein diets	Rockland chow–low protein diets
	Vit. B_{12} or saline, 21 pr* & 6 p days (1)	Vit. B_{12} or saline, 6 p days (2)	Diff. between pr & p, & only p admin. (1)–(2)	Vit. B_{12} or saline, 6 p days (3)	Vit. B_{12} or saline, 21 pr* & 6 p days (4)	Vit. B_{12} or saline, 6 p days (5)	Diff. between pr & p, & only p admin. (4)–(5)	Vit. B_{12} or saline, 21 pr* & 6 p days (1)–(4)	Vit. B_{12} or saline, 6 p days (2)–(5)	Vit. B_{12} or saline, 6 p days
Vit. B_{12}										
Avg tensile strength	125.2	133.3	—8.1 (b)	129.5	82.0	89.4	—7.4 (b)	43.2 (a)	43.9 (a)	40.1 (a)
No. of rats	7	7		10	8	10				
Infection rate (%)	0	0		29	22	50				
Saline controls										
Avg tensile strength	102.0	111.9	—9.9 (b)	108.2	76.5	78.4	—1.9 (b)	25.5 (a)	33.5 (a)	29.8 (a)
No. of rats	6	7		10	7	15				
Infection rate (%)	0	16.6		20	54.5	39				
Difference (Vit. B_{12} — saline)	23.2 (a)	21.4 (a)		21.3 (a)	5.5 (b)	11.0 (b)				

Note: All wounds tested on 6th p day. Designated diets given for 21 pr and 6 p days. Vit. B_{12} dosage 0.5 μ/100 g body wt/day. Isotonic saline dosage was wt of rat/200. Student's t-test at .01 levels used to determine significance. (a) designates that the difference is statistically significant. (b) the difference is not statistically significant.

* pr = pre-operative; p = post-operative.

per 100 g of diet. The rest were maintained on the Rockland Chow diet said to contain 25% protein by analysis and with a caloric value of 320 calories per 100 g of diet. The weights of the animals were recorded daily and average daily intakes of food were determined. Percentages of weight change during the pre and postoperative periods were calculated (Table I). The rats on all these diets were given subcutaneously 0.5 μ of vit. B_{12} per 100 g of body weight in saline daily, while the controls received a comparable amount of saline daily. These solutions were given to some animals for the entire experimental period and to others only following operation (Table II). Except as noted later, all rats were killed on the sixth postoperative day. At this time the incidence of wound infection was noted (Table II). The wounds of the non-infected animals were excised, divided into 1 cm strips and tested for tensile strength as previously described(6). From each wound at least 3 segments were tested by a tensionmeter and the results were measured in grams. The strengths of all strips taken from a wound were totaled and divided by the number of segments. This gave the average tensile strength of the wound per cm. Sections were taken for microscopic study.

Results. All rats gained weight on the balanced synthetic and Rockland Chow diets and those fed low protein diets for 3 weeks invariably lost weight. Giving vit. B_{12} neither increased the food intake nor did it alter the weight changes in any group. This was evident in both the pre and postoperative periods (Table I).

Vit. B_{12} given either before and after operation or only postoperatively caused an increase in the tensile strength of sixth day wounds in rats fed the synthetic diet containing 19% protein, and the same effect was noted when B_{12} was given postoperatively to Rockland Chow fed animals. No difference was observed in the average tensile strengths for groups given the vitamin both before and after surgery, or only after operation. The tensile strengths of wounds in animals maintained on the low protein diet showed a significant retardation of wound healing when compared to those fed the 19% or 25% protein diets. The administration of B_{12} to the low

protein rats caused no significant increase in the strengths of their wounds. For results of statistical analysis of the above differences, see Table II. The low protein rats in general had a high incidence of wound infection by gross examination. (Table II). Vit. B_{12} did not check this complication.

Sections of the wounds studied by microscopy showed the usual findings for normal and low protein fed animals. The latter showed diminished fibroplasia, derangement of the normal polarity of the fibroblasts and evidence of tissue edema. The administration of vit. B_{12} did not cause microscopic changes in the rats of any group.

Since vit. B_{12} when given to rats maintained on balanced diets increased on the average the tensile strength of six-day-old wounds, it seemed important to know when this increased strength was acquired. Accordingly, studies were made on wounds three to eight days old in rats fed balanced synthetic or Rockland Chow diets. Postoperatively they were given parenteral vit. B_{12} or saline from the time of operation until death. With the animals on these diets, statistical analysis did not show any difference in the average change of tensile strength from the third to the eighth day when comparing the vitamin groups with the saline controls (Table III). It was determined, however, that the average tensile strength was significantly higher on the third day in the vitamin fed groups. Thus the stronger wounds previously noted at the sixth day in rats fed B_{12} could possibly be explained by greater acquisition of tensile strength by the third day. This would indicate that the latent period of healing had been shortened in the rats given B_{12}. The continued greater acquisition of strength, on the other hand, was not evident by the eighth day when the vitamin and saline groups were compared. In these two groups, more extensive data will be necessary to detect a significant difference in the change of average tensile strength from the third to the eighth day.

TABLE III. Effect of Vit. B_{12} on Mean Tensile Strength of Wounds in Rats Fed Balanced Diets from Third to Eighth Day (See Text).

Post-op days tested	Rockland chow diet: No. tested	Rockland chow diet: Tensile strength (g)	Balanced synthetic diet: No. tested	Balanced synthetic diet: Tensile strength (g)
		Vitamin B_{12}*		
3	3	54.9	4	51.8
4	4	68.7	4	60.7
5	6	80.8	5	96.6
6	10	129.5	7	133.3
7	7	152.4	6	139.8
8	5	170.0	5	173.5
		Saline†		
3	3	39.7	4	42.6
4	3	62.0	6	60.3
5	5	80.7	5	81.8
6	10	108.2	7	111.9
7	5	140.0	6	127.4
8	5	159.7	5	179.6

* 0.5 μ/100 g body wt/day.

† Dosage of isotonic saline was $\frac{\text{wt of rat}}{200}$.

Respective diets were given 3 wk before surgery and postoperatively until animal was killed.

Summary. 1) These data show that B_{12} increased the tensile strength of wounds during the early phases of healing in rats fed balanced diets containing 19% or 25% protein. This effect was evident at least by the third day in the wounds studied, and it was most noticeable by the sixth day. 2) The wounds that were studied in the saline control and vitamin treated rats from the third to the eighth day showed no significant difference in their healing rates at the eighth day. 3) The delayed healing rate and increased incidence of wound infection in rats that were protein depleted by diet were not significantly altered by administration of vit. B_{12}.

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Parathyroid Extract and Alkaline Phosphatase Activity.* (20157)

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Parathyroid extract has been reported to increase(1) and to have no effect(2) on the alkaline phosphatase activity of the kidneys of rats. This difference now has been resolved by repeating the experiments with each of the separate laboratories performing simultaneously both of the previously reported procedures.

Results. The results obtained in both laboratories were identical (Table I). The parathyroid extract‡ produced an increase in phosphorus excretion, but no change in the

TABLE I. Inability of Parathyroid Extract to Change Alkaline Phosphatase Activity of Kidney.* (5 male rats per group.)

Treatment	B. wt, g	Urine P, mg (24 hr)	Kidney, g	Alkaline phosphatase, U/g — Homogenate at 5°, 18 hr† — K–A pH 9.7	K–A 8.6	Bodansky 9.7	Bodansky 8.6
			Bonelli and Sala				
Control	215 ±10‡	5.1 ± 1.7	1.64 ± .10	56 ± 3.4			35 ± 2.2
PTH 1 ml	200 ± 6	9.6 ± 1.5	1.52 ± .05	54 ± 6.4			32 ± 1.7
			Kochakian and Reed				
Control	209 ± 6	7.7 ± 1.7	1.52 ± .07	111 ± 5.0	46 ± 4.9	36 ± 5.2	19 ± 2.1
PTH 2 ml	210 ± 6	24.1 ± 2.6	1.51 ± .04	113 ± 4.4	46 ± 2.9	34 ± 3.5	18 ± 1.3
				Homogenate at 23–30°, 42 hr† — K–A pH 9.7	K–A 8.6	Bodansky 9.7	Bodansky 8.6
			Bonelli and Sala				
Control	215 ±10‡	5.1 ± 1.7	1.64 ± .10	18 ± 4.2			18 ± 4.2
PTH 1 ml	200 ± 6	9.6 ± 1.5	1.52 ± .05	19 ± 2.2			13 ± 3.4
			Kochakian and Reed				
Control	209 ± 6	7.7 ± 1.7	1.52 ± .07	89 ± 3.9	36 ± 2.1	29 ± 3.1	25 ± 1.7
PTH 2 ml	210 ± 6	24.1 ± 2.6	1.51 ± .04	75 ± 4.0	34 ± 5.7	27 ± 4.9	22 ± 2.4

* Autopsy 6.5 hr after inj. of parathyroid extract.
† Temperature and duration of autolysis.
‡ Stand. error of the mean, $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

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† Part of this investigation was aided by a grant from the American Cancer Society.
‡ The parathyroid extract was provided by Eli Lilly and Co.

alkaline phosphatase activity of the kidneys. The larger dose of the parathyroid extract produced a greater excretion of phosphorus(1). The method of autolysis of the homogenate, the type of substrate and the pH of the enzyme determination did not reveal any differences between the two groups of animals. Similar results were obtained in rats studied at 2, 4, 6, and 8 hours (Bonelli and Sala) and 6 hours (Kochakian and Reed) after an injection of 1 ml of parathyroid extract.

The divergent findings previously reported (1) were very likely due to observations based on an insufficient number of animals.

It should be noted that greater enzyme activities were obtained at pH 9.7, by the King-Armstrong (K-A) method, and by autolysis at 5°.

Conclusion. Parathyroid extract produces an increased excretion of phosphorus, but no change in the alkaline phosphatase activity of the kidney of male rats.

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Influence of Administration of ACTH on Urinary Amino Acids.* (20158)

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The present paper describes some changes produced by the administration of ACTH‡ on the urinary output of nitrogenous substances with particular emphasis on amino acids. Urine samples were obtained during 3 periods of study in 2 female psychotic patients. Since the results for all 3 collection periods were similar, the results for only one will be discussed in detail. Both of these patients responded in a similar manner to each other and as reported for normal subjects in the literature and other psychotic patients tested in this laboratory(1) with regard to drop in eosinophils, sodium retention, potassium diuresis, and negative nitrogen balance (see Fig. 208 of reference (1) for pertinent data on patient reported). A preliminary report has been made of this work(2).

Methods. The data reported are for a 29-year-old schizophrenic female who was studied for 4 days prior to administration of the hormone, 10 days during the administration of a daily dose of 150 mg of ACTH, and for 5 days after the cessation of the treatment. The hormone was administered 6 times daily in 25 mg doses via the intramuscular route. The various *nitrogenous constituents* listed in Table I-A were determined by customary procedures. The microbiological determinations shown in Table I-B were performed on urine dialysates by previously described methods(3). For *2-dimensional paper chromatography* samples of urine were dialyzed to equilibrium against 3 ml of glass-distilled water in a rocking dialyzer for 18-24 hours at 4°C. Samples corresponding to 1×10^{-4} of the 24-hour excretion were employed for chromatography. Aliquots of the dialysate were hydrolyzed for 18-24 hours in 6 N HCl in a sealed tube at 110°, and the acid removed *in vacuo* prior to placing the samples on paper. Two-dimensional chromatograms were made of the protein-free dialysates before and after acid hydrolysis by the method of Consden *et al.*(4), as extended by Dent(5,6). By visual inspection it was possible to compare the 24-hour excretion of the major free and bound amino acids from day to day. Urine

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‡ The adrenocorticotropic hormone used in these experiments was furnished by Armour and Co. through the courtesy of Dr. John Mote.

TABLE I. Influence of ACTH on Distribution of Urinary Nitrogenous Constituents. Results in g/Hr under A; mg/Hr under B.

	Constituent		Control	ACTH (150 mg/day) Day 4	Day 8	Day 10	Recovery*
A							
	Total N		10.0	11.4	18.1	17.9	11.1
	α-amino N		.10	.41	.44	.43	.08
	Ammonia N		.28	.63	.99	.77	.23
	Urea N		8.72	9.00	14.56	14.90	9.88
	Uric acid N		.20	.29	.43	.41	.19
	Creatinine N		.32	.32	.30	.31	.29
	Undetermined N		.38	.75	1.38	1.08	.44
B							
	Aspartic acid	F†	4	11	22	19	7
		C†	133	210	340	285	120
	Glutamic acid‡	F	30	31	46	50	23
		C	322	460	660	530	373
	Threonine	F	23	61	287	313	22
		C	22	58	26	0	21
	Histidine	F	158	305	526	520	100
		C	tr§	tr	tr	tr	tr

* Second day after last inj. of ACTH.

† F = free; C = combined. Values for "free" amino acids represent microbiological response before acid hydrolysis, while "combined" values represent the difference between results obtained after and before acid hydrolysis.

‡ Prior to analysis the samples were autoclaved under conditions leading to virtually complete destruction of glutamine.

§ tr = trace.

samples obtained during the entire period of study were examined by these methods.

Results. The results in Table I-A show the nitrogen distribution in the urine during typical control and recovery days and for 3 days during the period of daily administration of 150 mg of ACTH. All of the substituents studied except creatinine increased during the administration of the hormone. The proportionate increases in α-amino nitrogen and ammonia were greater and occurred before marked elevations were noted in the excretion of total nitrogen, urea, or uric acid. It was found that the total α-amino acid nitrogen of post-absorptive plasma was not significantly increased by hormone administration. The levels were 5.1 and 5.2 mg % on the 4th and 12th days of hormone treatment, respectively, as compared to the control value of 4.8 mg %. The possibility is not ruled out that during hormone treatment the plasma levels of amino acids might have been significantly higher during active absorption of protein split-products after a meal, with the consequent excretion of those substances for which the renal threshold was exceeded for a short time. It has been shown by Dent and Shilling(7) that after the ingestion of proteins large rises occur in the concentrations of many amino acids in the portal blood of dogs and that there can be an accompanying amino aciduria. The increase in ammonia excretion occurred at the time that sodium retention began to exceed chloride retention, and could presumably be a result of the response of the kidney to increased acidity of the urine. During the first 4 days of ACTH administration there was a retention of 280 meq. of sodium and a loss of 40 meq. of potassium, or a net retention of 240 meq. of base, while the chloride retention was only 100 meq. On the 4th day only 10% of the ingested sodium was excreted, while 90% of the potassium and 70% of the chloride appeared in the urine. At this time the pH of the blood had increased from 7.3 to 7.6 and the pH of the urine had dropped from 6.8 to 5.0.

Chromatograms of a urine sample before and after hydrolysis for a typical control day are shown in Fig. 1 and 2. Glycine was present in the highest concentration in the free form. Serine, alanine, glutamine, taurine,

Key to numbers on chromatograms: Leucines, 1; tyrosine, 2; valine, 3; α-amino-n-butyric acid, 4; taurine, 5; cystine (cysteic acid), 6; histidine, 7; alanine, 8; threonine, 9; β-alanine, 10; glutamine, 11; glycine, 12; serine, 13; asparagine, 14; glutamic acid, 15; aspartic acid, 16; lysine, 17; X_1–X_5, unidentified substances. Some substances discussed in text were excreted in such small quantities that the spots did not appear on photographs, although they were easily detectable on original chromatograms.

FIG. 1.

cystine, and glutamic acid were present in appreciable, but smaller amounts. In some of the samples there were detectable quantities or traces of histidine, α-aminobutyric acid, γ-aminobutyric, valine, the leucines, tyrosine, lysine, threonine, β-alanine, and aspartic acid. Upon acid hydrolysis there were large increases in the amounts of glutamic and aspartic acids, glycine, and β-alanine. Increases in these amino acids on hydrolysis of urine have been reported by Dent(6,7).

During the period of administration of the hormone there were increases in the excretions of a number of the constituents in the free form. This effect was noted even in the first sample collected after the beginning of administration of the hormone, but appeared to reach its maximum intensity at 3 to 4 days after beginning of treatment, with the high level of free amino acid excretion continuing throughout the entire period of administration of the hormone. A typical experimental day is represented by the chromatograms obtained from the urine on the fifth day after the first administration of the hormone (Fig. 3 and 4). A comparison of Fig. 1 and 3 reveals that especially marked increases were found in free glycine, serine, alanine, taurine, glutamine, threonine, and lysine. The contents of cystine, histidine, and glutamic and aspartic acids were also somewhat increased. Of particular interest was the appearance of relatively large amounts of asparagine, an amino acid not found in the control samples. This amino acid was identified by the characteristic orange-brown color, the position on the chromatogram, the destruction on acid hydrolysis, and by the finding that known samples of

FIG. 2.

FIG. 1 and 2. Chromatograms of unhydrolyzed and hydrolyzed urine samples obtained during a control day.

asparagine, when added to the urine samples, migrated to the same position as the substance in urine and intensified the color. As in the controls (Fig. 2), there were marked increases in the content of glutamic and aspartic acids and β-alanine after acid hydrolysis of the urine obtained during the hormone treatment (Fig. 4). However, almost all of the glycine was now found in the free form, little or no increase in glycine being found on acid hydrolysis. It is interesting in this connection that there was a decreased utilization of the nitrogen of N^{15}-labeled glycine for protein synthesis in normal and adrenalectomized rats receiving cortisone(8) and a decreased incorporation of C^{14}-labeled glycine in hypophysectomized rats receiving ACTH(9). There was a precipitous drop in free amino acid excretion on the first day after cessation of administration of the hormone to levels slightly below those found during the control period (Fig. 5), together with a disappearance of asparagine from the urine. After acid hydrolysis (Fig. 6) the pattern of amino acids was very similar to that of the controls. Chromatograms made of samples obtained on the following 4 days showed patterns virtually identical with those found during the control period, both before and after acid hydrolysis. It thus appears that the influence of the hormone on amino acid excretion is rapidly reversible.

The large increase in the glutamine content of the urine and the appearance of relatively large amounts of asparagine during the administration of the hormone are particularly interesting. Although there is much information concerning the metabolism of glutamine in animal tissues(10,11) there is little comparable information about asparagine. Dent (6) has reported the presence of asparagine in "pathological urine", although the nature of the pathological states was not specified. The

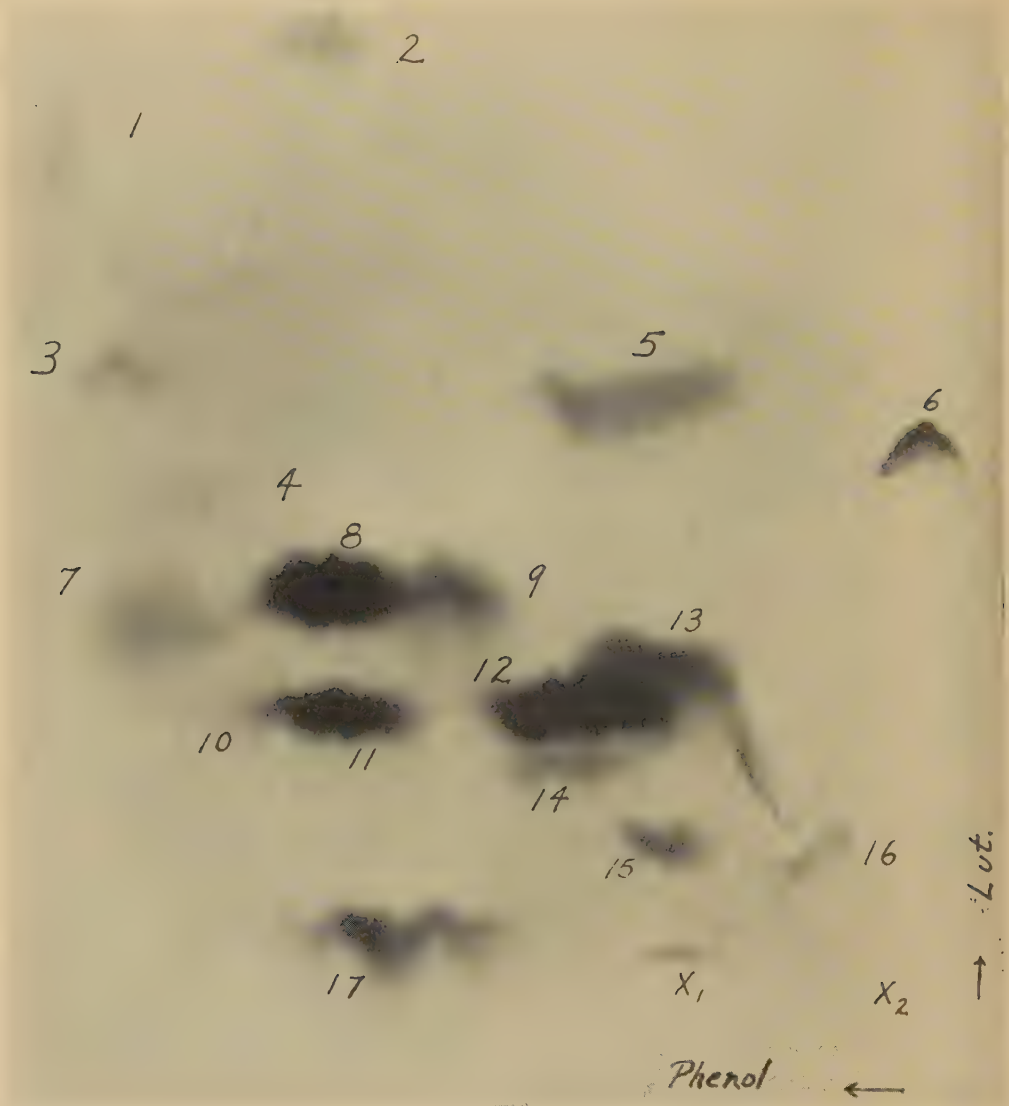

FIG. 3.

recent finding that glutamine and asparagine play a significant role in transamination in liver(12) is of particular interest in this connection, and a study of the influence of the administration of ACTH and cortisone on these processes is suggested. It does not appear likely that the increased excretion of glutamine in the present instance is caused by a diminished rate of its utilization for the formation of urea and ammonia, since there was actually an increased urinary excretion of both of these substances during administration of the hormone (Table I-A).

The microbiological determinations of 4 amino acids (Table I-B) are in agreement with the chromatographic findings. Increases of threonine and histidine were almost entirely in the free form, while bound forms of glutamic and aspartic acids accounted for the chief increment in the latter amino acids. Consideration could not be given to the microbiological availability of the bound forms of the amino acids, since their chemical nature was not known. However, the above results indicate that the bound forms of aspartic and glutamic acids are largely not available to the

FIG. 4.

FIG. 3 and 4. Obtained on fifth day after first administration of ACTH.

test organisms. The values for free threonine and histidine found during the control period are in close agreement with those previously reported for normal individuals(13). However, the maximal increases in the excretion of these amino acids upon administration of ACTH were much greater in the present study than in the one cited above(13). This may possibly be attributable to the fact that a 40 mg daily dose of ACTH was employed in the latter study as compared to 150 mg in the present experiments.

Discussion. The results reported herein are in keeping with those previously cited(8,13, also see bibliography in these references) which indicate that the administration of ACTH or cortisone can produce profound alterations in nitrogen metabolism. The multitude of alternative pathways, both physiological and biochemical, which might be affected prevents fruitful speculation at the present time about the mechanism of action.

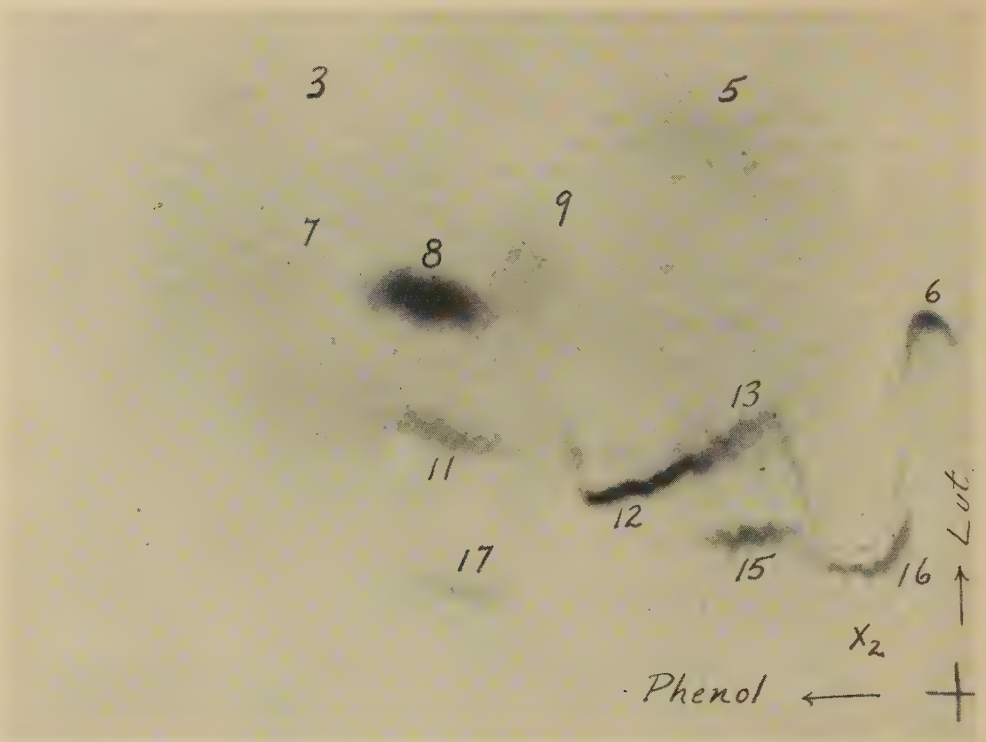

FIG. 5.

Considerable evidence(14) has been adduced for the conclusion that optimal utilization of amino acids for protein synthesis "requires the simultaneous availability of adequate amounts of all of the essential and non-essential amino acids participating in the make-up of the given protein". It would be expected that losses of essential and non-essential amino acids such as were found in the present study would decrease the potentialities for protein synthesis at various sites in the body.

A study of the influence of cortisone on the patterns of free amino acids in the tissues of the developing chick embryo showed that the chief abnormality produced by the hormone was an accumulation of free hydroxyproline (15). In the present study the latter amino acid was not detected in the urine either during the control period or during the administration of ACTH.

Summary. Typical results are presented showing the influence of ACTH administration in the human on the urinary excretion of nitrogenous substances. The hormone evoked notable increases in the excretion of amino acids, ammonia, urea, uric acid, and some undetermined nitrogenous constituents. Two-dimensional chromatograms revealed some of the detailed changes in the excretion of both free and bound amino acids. Microbiological determination of some of the amino acids gave results which were concordant with the chromatographic findings. All of the urinary nitrogenous constituents returned to normal levels shortly after cessation of the treatment. The possible significance of some of the findings is indicated.

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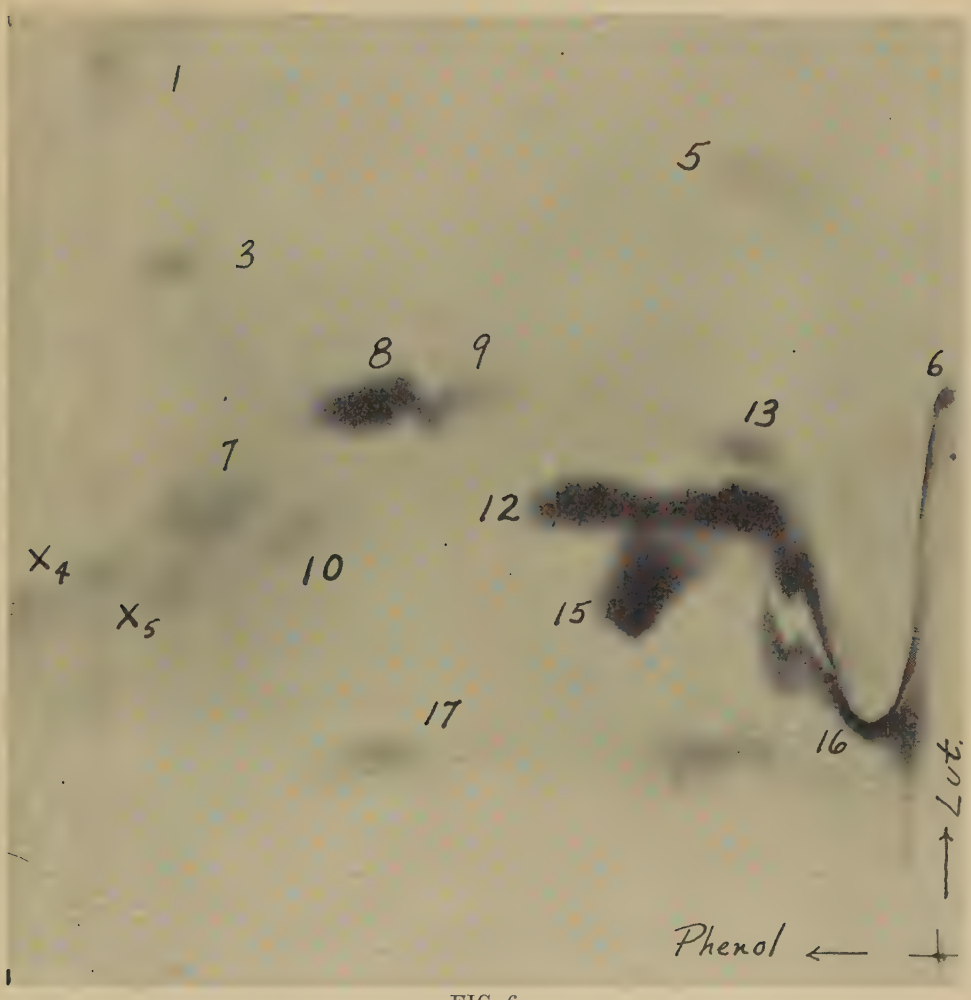

FIG. 6.

FIG. 5 and 6. Obtained on first day after cessation of administration of ACTH.

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The Effect of Inanition on Liver Catalase Activity in Mice.* (20159)

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Greenstein(1) reported that a 3-day starvation period caused no decrease of rat liver catalase, whereas Miller(2,3) showed a definite lowering of the liver enzyme level in rats after a 7-day period. Rats on a protein-free diet for 7-10 days had a liver catalase activity of approximately 50% of normal(4). Begg (5) has recently reported that force-fed, tumor bearing rats still showed a decrease in liver catalase. The present study shows an effect of food and water withdrawal on mouse liver catalase.

Methods. Twenty C3H mice and 26 C57 black mice, 3 to 4 months of age of both sexes, were maintained on Purina Laboratory Chow and water *ad libitum*. Since the animals often reduced their food consumption when taken out of their regular cages, it was measured for 3 days before the experimental period. The mice were then separated into the following categories: 1) animals receiving food and water *ad libitum*, 2) animals receiving food *ad libitum* with water withheld, 3) animals receiving water *ad libitum* with food withheld, and 4) animals receiving neither food nor water. The group was maintained in this manner for 24 hours, after which they were killed. Catalase activity in the livers was determined by our modification(6) of the manometric method of Perlmann and Lipmann(7). Calculations were made on both a wet weight and a dry weight basis. Erythrocyte catalase had no significant effect since it is much less active than liver catalase. The values are reported as the evolution of O_2 per 44 x 10^{-8} g of liver, which is the actual amount of tissue used for assay.

Results and discussion. Experimental results are summarized in Table I. Following a 24-hour period of food and water deprivation, liver catalase was lowered by approximately one-fourth in both strains. A somewhat greater lowering occurred in the C3H strain, which had higher normal values. The difference between groups receiving water but no food and the group receiving neither, was not statistically significant.

The decrease in animals deprived of water alone was about one-half of that in animals deprived of food or food and water, probably because they voluntarily restricted their intake to about one-half the normal.

Summary and conclusions. 1. A 24-hour withdrawal of food and/or water results in a lowering of liver catalase activity in mice of both C3H and C57 black strains. 2. Liver

TABLE I. Effect of Inanition on Liver Catalase Activity.

Strain	No. of mice	Diet	Catalase activity: Wet tissue	Catalase activity: Dry tissue	Avg of % of normal food intake
C3H	5	Food and water	2.15 ± .34	6.98 ±1.24	
57b*	7	*ad lib.*	1.61 ± .14	4.89 ± .42	
3H	5	Food *ad lib.*,	1.91 ± .22	5.94 ± .70	51.8% ± 7.6
57b*	7	no water	1.50 ± .19	4.42 ± .60	63.1% ± 13.4
3H	5	Water *ad lib.*,	1.69 ± .22	4.81 ± .67	
57b*	5	no food	1.31 ± .15	3.52 ± .48	
3H	5	No food, no	1.70 ± .17	4.75 ± .51	
57b*	7	water	1.35 ± .11	3.71 ± .40	

* b = black.

* Aided by grants from the Cancer Control Branch of the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, and the Alabama Division, American Cancer Society.

catalase measurements in tumor bearing or treated mice are significant only if food and water intake are controlled.

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Effect of Complete Hepatic Vein Ligation on Portal Pressures and Ascites Formation in Dogs with Porta-Caval Shunts.* (20160)

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Bollman(1) stated, "Because of the anatomic relation of the liver to the inferior vena cava, no suitable technic has been devised for direct constriction of the hepatic veins." Simonds and Brandes(2) and Brandes(3) studied the effects of obstructing the hepatic veins by use of a mass ligature technic employing a soft rubber tube as a tourniquet to occlude the veins. Child and associates(4) occluded the hepatic veins in the *Macaca mulatta* monkey by placing a polythene tube in the inferior vena cava held in place by a ligature above and below the entrance of the hepatic veins. Most investigators have relied on supra-diaphragmatic constriction of the inferior vena cava to produce increased intrahepatic and portal pressures in the study of ascites production. The purpose of this study was to develop a technic for complete individual occlusion of the hepatic veins, and to determine the effects of such occlusions on portal pressures, liver function, pathologic changes in the liver, and ascites formation.

Methods. Healthy mongrel dogs were used. Preliminary efforts showed that total ligation of the hepatic veins resulted in 100% mortality. Immediately after occlusion of the last hepatic vein the inferior vena cava collapsed almost completely and the animals died in acute shock from pooling of blood in the portal system in a period of 5 to 30 minutes. If the compression of the portal outflow was released just before the heart stopped, the dogs recovered completely. In each case the last vein to be occluded was the large proximal vein just below the diaphragm which drains all of the liver except the right lateral lobes (Fig. 1). If this vein was left untied, dividing all of the others, the dogs survived indefinitely with no ill effects. To attain survival in dogs with total ligation of the hepatic veins a porta-caval shunt had to be done in conjunction with the ligation. This allows portal blood to flow directly into the inferior vena cava, and the hepatic artery inflow to the liver then escapes into the inferior vena cava in a retrograde manner through the proximal segment of the portal vein and the porta-caval anastomosis. Fifteen dogs weighing 11 to 22 kg were prepared and studied in this manner. A thoraco-abdominal approach through the right tenth interspace, dividing the diaphragm from its costal attachment to the hiatus of the inferior vena cava, was employed to divide the hepatic veins. The vena cava in this area not only is intimately adherent to the diaphragm, but also is surrounded by liver, and must be dissected from these attachments before the division of the individual hepatic veins is begun (Fig. 2).

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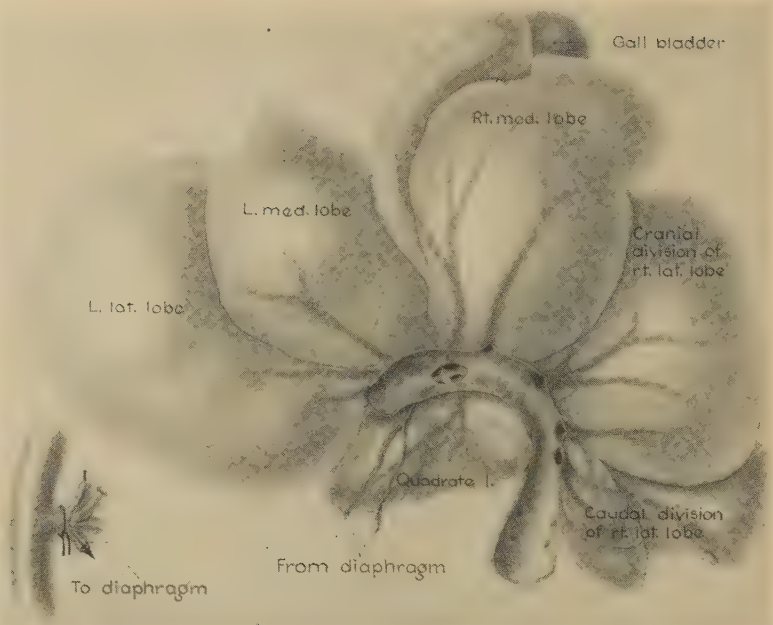

FIG. 1. Drawing illustrating major hepatic veins emptying into the inferior vena cava and areas of liver drained by them. Note diaphragmatic vein emptying into large proximal hepatic vein. Inset shows the possibility of collateral circulation behind a ligature placed on this vessel.

The dissection is started distally separating the inferior vena cava from its investing sheath and is then carried proximally by gently dissecting the vena cava from its liver bed dividing the hepatic veins between ligatures of 2-0 silk as the veins are encountered. The 2-0 ligatures are reinforced with stick ties of 3-0 silk. There are innumerable small vessels present in addition to the 5 large veins shown in Fig. 1. This part of the dissection is made difficult by the fact that the hepatic veins are large and bifurcate very close to their junction with the inferior vena cava. To give added length to the veins the liver substance is dissected back for a short distance along the veins. If the hepatic veins are divided too close to the inferior vena cava, part of the wall of the latter vessel is included in the ligature, which not only partially occludes the lumen of the inferior vena cava, but also increases the hazard of slipping of the ligatures. The large proximal vein is merely ligated instead of ligated and divided because of its size and difficulty of exposure. Before this last vein is occluded, a side to side porta-caval anastomosis is done utilizing a single Pott's clamp. The full available width of the clamp is used resulting in an anastomosis measuring 1 x 2 cm. When the procedure outlined above is completed there is a possibility for collateral venous drainage from the liver to the diaphragm present behind the ligature on the large proximal vein as shown in the insert in Fig. 1. If this small diaphragmatic vein is obstructed at the time the hepatic veins are ligated, the operative mortality approaches 100% even with an adequate porta-caval shunt. However, its value as a collateral diminishes as readjustments in the circulation take place because this vein was filled with clot in 14 of the 15 dogs in this study at the time of autopsy. Post-operatively the dogs were maintained on a standard kennel diet with whole milk supplement. Liver function and blood studies were done periodically, the onset and amount of ascites recorded, and the animals reexplored transabdominally to obtain portal pressures and liver biopsies after 2 to 5 weeks. Portal pressures were done in a branch of the right gastro-epiploic vein. The animals were followed for periods of 2 to 28 weeks. At autopsy the porta-caval shunts ranged from .7 x .9 cm to 1 x 3 cm. The majority were 1 x 2 cm.

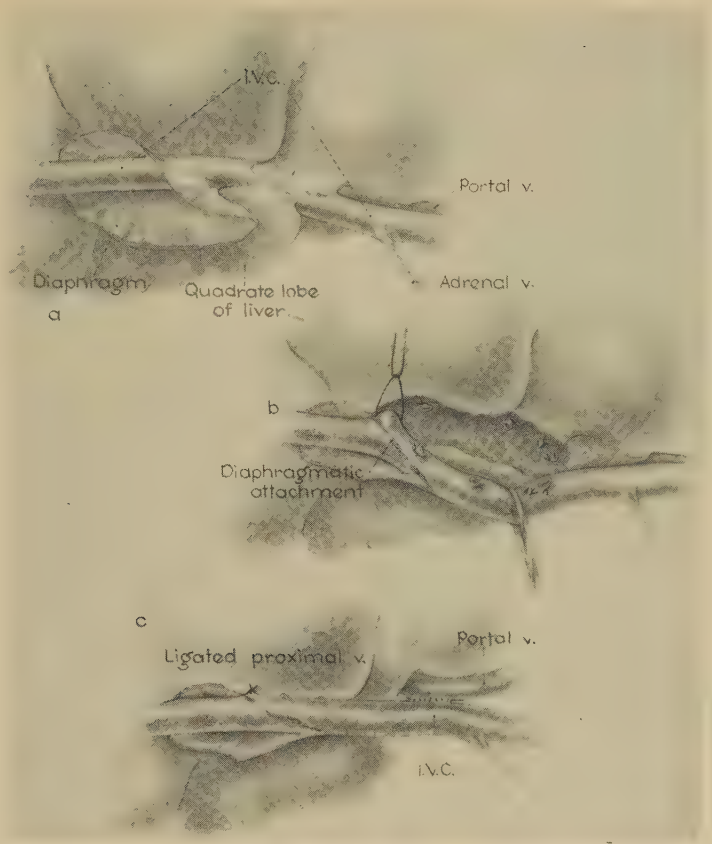

FIG. 2. Outline of main steps in individual occlusion of hepatic veins in conjunction with a porta-caval anastomosis. A. Inferior vena cava exposed showing its intimate relationship to the diaphragm and dorsal surface of the liver. B. Inferior vena cava has been dissected from its liver bed by ligating and dividing all hepatic veins except the most proximal vein. The porta-caval anastomosis is done at this stage. C. Porta-caval anastomosis completed, and ligature tied down around the large proximal vein. Major hepatic veins are much larger than represented here, and there are also numerous small veins which have to be ligated and divided.

Results. Effects on portal pressures and ascites formation. There was no uniformity in the development of increased portal pressures or ascites (Table I). Portal pressures were measured in the 15 dogs and ranged from 11 to 68 cm of water. Pressures in control dogs ranged from 11 to 15 cm. Ten dogs or 66% had portal pressures that were elevated above 19 cm of water. Four dogs or 27% had pressures from 15 cm to 19 cm, and one dog had a pressure of 11 cm of water. Eight or 53% of the 15 dogs in which portal pressures were measured developed ascites high in protein content which became apparent within 2

TABLE I. Effects of Total Hepatic Vein Ligation in Dogs with Porta-Caval Shunts.

Dog No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Portal pressure, cm H_2O	11	15	15	16	17	19	20	22	26	28	32	42	43	58	68
Ascites	+	—	+	—	—	+	—	+	+	—	+	—	—	+	+
Central necrosis & congestion	2+	+	2+	2+	+	3+	+	2+	3+	3+	3+	3+	2+		4+
Serum albumin, g/100 cc	1.6	2.2	1.7	1.6	1.7	2.8	2.3	1.8	1.8	1.6	1.5	2.1	1.7	3.8	2.0
Total serum protein, g/100 cc	5.0	5.3	4.7	6.5	7.1	5.2	6.2	5.5	4.7	6.3	4.7	6.4	6.4	4.3	4.9
% BSP* retention	17	7	6	6	7	6	5	12	7	7	9	19	6	5	8

* Control dogs show less than 6% retention in 45 min.

FIG. 3. Dog 11 showing marked ascites and collateral circulation in abdominal wall. This dog formed 22 liters of ascitic fluid over a period of 3 months and required repeated paracentesis.

weeks. The ascites varied from 200 cc in one dog to 22 liters in another, the latter amount of fluid being removed over a period of 3 months. The total proteins in the ascitic fluid averaged 3.6 g/100 cc with an albumin content of 1.0 g %. Those animals with marked ascites showed collateral vessels in the abdominal wall (Fig. 3). No esophageal varices or large collaterals in the region of the diaphragm were observed.

Laboratory studies. The blood sugar, blood urea nitrogen, and serum bilirubin were normal in all dogs. The hemoglobin values ranged from 7.8 to 16.0 g. Serum protein levels were low in general, especially the albumin fraction. These values are shown in Table I, along with the bromsulfalein clearance values.

Pathology. Grossly the livers were large and congested with rounded edges. Exudate and adhesions were present over the surface. There were dilated lymphatics and large lymph nodes in the hilar region. None of the dogs used in this study showed residual hepatic veins emptying into the inferior vena cava at the time of autopsy, and there was no instance of incomplete ligature or recanalization of the large proximal vein. Clots in various stages of organization were present in the hepatic veins at the site of their occlusion. Microscopically the sinusoids were dilated and filled with blood or amorphous pink staining material. Varying degrees of central necrosis and degeneration of the liver cell cords were found ranging from minimal changes to a marked degree of destruction. No signs of fibrosis or regeneration were present. In some sections thickened capsule and dilated subcapsular lymphatics were seen.

Discussion. This is the first time that survival following occlusion of the hepatic veins has been reported. Child, McClure, and Hays(4) observed survival in monkeys up to 3 hours following supportive treatment. Complete occlusion of the hepatic veins results in marked congestion of the liver with subsequent death from shock due to pooling of blood in the portal system. The porta-caval shunt acts as an avenue of escape permitting the blood in the liver to leave the portal bed. This retrograde blood flow through the porta-caval anastomosis can readily be seen at the conclusion of the anastomosis and may be demonstrated more clearly by temporarily occluding the portal vein on the intestinal side of the porta-caval shunt. The presence of free communications between the arterial and portal venous systems in the liver is thus established by these experiments.

There was no correlation between the size of the porta-caval shunt and the subsequent elevation of portal pressure; in fact, the dog with the smallest shunt in the series did not develop either an elevated portal pressure or ascites. It is difficult to explain such findings, but the work of Madden(5) and his associates gives some insight to this problem. These investigators have shown by injection studies in human cadavers that pre-existing natural porta-caval shunts were present in 4 out of 8 cadavers studied. Such pre-existing shunts may also explain the wide variations in portal pressures in the dogs studied in this experiment.

Volwiler and associates(6,7) stress the importance of hepatovenous congestion and increased liver lymph flow, and McKee *et al.*(8) have shown the effect of serum protein and salt on ascites production. In the present study an attempt was made to correlate the development of ascites with an increase in the portal vein pressure, a decrease in serum albumin, or with the degree of liver damage as shown in the microscopic sections or the bromsulfalein test. Examination of Table I reveals that there was no consistent correlation found between any one of these factors and the degree of ascites formation in the dogs in this experiment. This finding would suggest that the formation of ascites more likely was due to a combination of factors rather than to any single one.

Summary. 1. A successful technic for individual ligation and division of the hepatic veins in the dog is described. 2. Complete occlusion of the hepatic veins is uniformly fatal in dogs. However, if a porta-caval shunt is also done, the dogs will survive. 3. Fifteen dogs prepared in this manner were studied, and 66% of them developed a significant elevation of the portal pressure. 4. Ascites developed in 53% of the dogs, but the ascites could not be correlated with the increased portal pressures, decreased serum proteins, or pathological changes in the liver.

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Some Relationships Between Intracranial Pressure and EEG Frequency in Cats.* (20161)

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This report describes some relationships existing between intracranial pressure and the EEG pattern in the curarized, unanesthetized cat.

Methods. Each cat was anesthetized with vinyl ether (Vinethene), and a wide midline skull exposure made. Four small holes were drilled through the skull, one over each parietal region and one over each side of the cerebellum. These holes were so placed as to center approximately over the motor cortices and cerebellar lobes. Screw-in type EEG electrodes were placed in these holes. Each animal was then tracheotomized and injected with dihydro-beta-erythroidin hydrobromide§ to eliminate muscle activity and then placed on artificial respiration. A number 18-gauge spinal needle was then inserted into the cisterna magna through the foramen magnum and the needle connected by means of a short rubber tube to a Statham strain gauge pressure transducer. The output of the transducer activated a high-gain amplifier coupled to a

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§ Courtesy Merck and Co., Rahway, N. J.

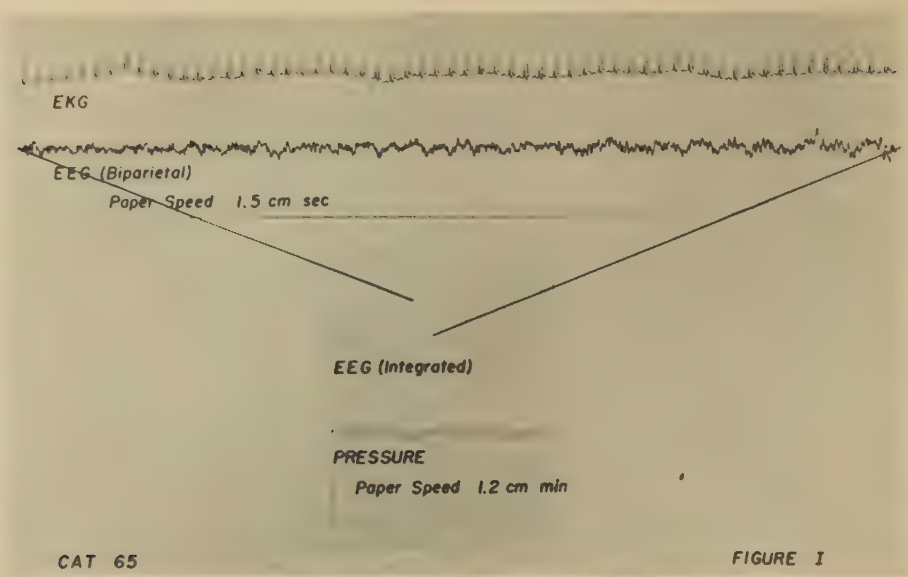

FIG. 1. Normal ambient atmospheric pressure. Decrease in EEG frequency is associated with increase in intracranial pressure, and vice versa.

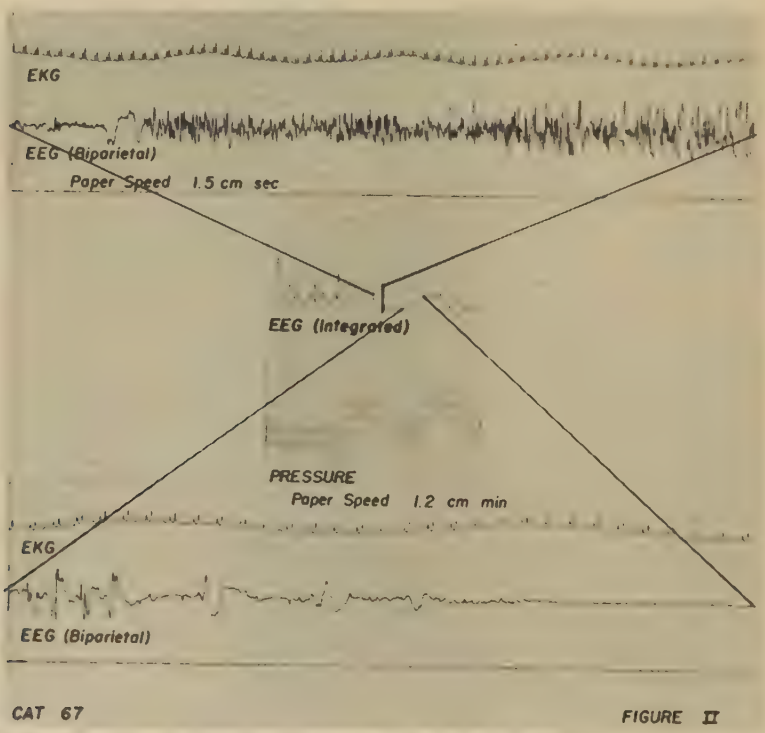

FIG. 2. Increased ambient atmospheric pressure. Decrease in EEG frequency is associated with decrease in intracranial pressure and vice versa.

power amplifier driving a Brush magnetic pen-motor. The entire pressure system was then filled with normal saline and calibrated against a water column of known pressure. Thus, a continuous ink-line representation of intracranial pressure was obtained. In about

one-third of our experiments, a brass tube of ¼-inch diameter was screwed into a threaded hole in the skull over the dura of the parietal region and connected directly to the pressure transducer.‖ Care was taken to avoid puncturing the dura. The pressure recordings obtained from the epidural space were similar to those obtained from the cisterna magna. Since the cisternal technic was simpler than the epidural, it was adopted as routine. Electroencephalograms were recorded on a Grass type III-B machine. In addition to the ink written electroencephalogram, a recording of the instantaneous average frequency of the brain waves was obtained by feeding the output from the Grass machine into a frequency analyzer designed by C. W. Goodwin. The output of the frequency analyzer was also recorded with a Brush magnetic pen-motor.

Results. With the animal exposed to normal ambient atmospheric pressure, control observations showed a quite consistent inverse correlation between the variations in EEG frequency and intracranial pressure. In 7 out of 8 experiments, a decrease in EEG frequency (25-30/sec to 15-20/sec) was associated with a rise (20% to 50%) in intracranial pressure, and vice versa (Fig. 1). In the eighth experiment, both the EEG frequency and the intracranial pressure varied directly with each other. When the animals were introduced into a pressure chamber where the ambient pressure as well as the partial pressure of inspired oxygen was increased(1), seizure activity was noticed in the EEG and a reversal in the relationship between EEG frequency and intracranial pressure took place. During the low frequency-high amplitude seizure activity period the intracranial pressure fell, and during the inter-seizure phases when the EEG reverted to high frequency-low amplitude activity, the intracranial pressure went up (Fig. 2). In only one experiment were these relationships reversed.

A few experiments were performed under conditions of normal atmospheric pressure in which seizures were produced by the intravenous administration of 1,5-pentamethylenetetrazole (Metrazol). The correlation between the two variables was the same as that observed during hyperoxic seizures.

Findings similar to ours were reported by Guillaume and Janny(2) in man. They made continuous intracranial pressure recordings which demonstrated rhythmic changes at a rate of 6 to 12 per minute; no EEG recordings were made.

Summary. 1. In the curarized, unanesthetized cat, the EEG frequency varies inversely with the intracranial pressure. 2. During seizures induced by high partial pressures of oxygen or by Metrazol, the intracranial pressure varies directly with the EEG frequency. 3. No explanation for our findings is offered at present.

We wish to thank Dr. Stephen L. Sherwood of the Middlesex Hospital, London, England, for valuable aid and suggestions.

‖ This device with amplifier kindly supplied by Dr. J. P. Marbarger and Mr. Clarence Pestel of the Aeromedical and Physical Environment Laboratory, University of Illinois, College of Medicine.

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Isolation from Mammalian Brain of Coproporphyrin III and a Uro-Type Porphyrin.* (20162)

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Klüver(1,2), using ultraviolet light and a direct vision spectroscope, was the first to describe the occurrence of porphyrin in the normal central nervous system of warm blooded animals. He found a sharp band with a maximum at about 625 mμ in the fluorescence spectrum and on this basis identified the substance as a coproporphyrin. He also confirmed this finding chemically but, being unable to obtain a melting point owing to an insufficiency of material, was not able to state whether the coproporphyrin was present as the type I or type III isomer. Later he tentatively identified it as a type III isomer(3). Since then the presence of coproporphyrin has been confirmed in this laboratory(4) and again the isomer was tentatively identified as coproporphyrin III, by means of the florescence quenching technic.

The finding of an increased excretion of coproporphyrin III in the urine of patients with poliomyelitis(5) raised the problem of the origin of this excess of porphyrin and its possible relation to the coproporphyrin of the central nervous system. Because of this and other more general considerations it was believed desirable to identify this porphyrin with certainty. The present report is concerned with the isolation of crystalline porphyrin from mammalian brains.

Methods. Fresh beef brains were used and extracted by the method of Schwartz and Wikoff for red blood cells(6). The brains were stripped of membrane, washed as clean of blood as possible, homogenized in a Waring blender and extracted in a ball-mill using a 4:1 ethyl acetate-glacial acetic acid mixture. In all 9.665 kg of brain were processed. The ethyl acetate-acetic acid extract was washed with 1% sodium acetate in a separatory funnel and the porphyrins were extracted with 3 N HCl. This was washed with ether to remove some of the liquid material which was invariably present and which interfered considerably with purification at all stages of the procedure. The 3 N HCl was then neutralized with saturated sodium acetate and, after the addition of a few ml of glacial acetic acid, shaken with ethyl acetate. This was washed with water and extracted repeatedly with 0.3 N HCl to remove the coproporphyrin. The latter solution was then diluted to 0.1 N and washed with chloroform to remove any traces of chloroform-soluble porphyrins. The 0.1 N HCl solution, after addition of sodium acetate and glacial acetic acid, was shaken with ether and the latter in turn extracted with 1.5 N HCl. The content of total coproporphyrin in the 1.5 N HCl, assayed fluorimetrically, was approximately 193 μg or 2 μg per 100 grams of brain, which is in rough agreement with previously published figures of the average content of human brain(7). The total coproporphyrin was concentrated in 3 N HCl and esterified by the addition of 10 volumes of a mixture of methyl alcohol-sulphuric acid (20:1) The porphyrin ester was purified by the method of Grinstein, Schwartz and Watson(8). Chloroform-petroleum ether was used to dissolve the residue and develop the chromatogram. Some lipid material appeared to be present throughout and caused a cloudiness in the chloroform-methyl alcohol used for attempted crystallization. This was successfully removed by suspending the porphyrin in petroleum ether and filtering. The porphyrin was then redissolved from the filter paper in chloroform and crystallized from methyl alcohol in the usual way(8). Coproporphyrin III methyl ester was obtained crystallizing slowly in the typical rosettes of straight needle-like prisms. On determining the melting point initially, shrinkage of the crystals was observed from 148°C onward with generalized melting at 174°C. Recrystallizations gave melting points of 146-149° and 146°C.

Results. During the initial phase of extraction the sodium acetate washings of the

* Aided in part under contract with the Atomic Energy Commission.

ethyl acetate were concentrated on aluminum oxide, eluted with 1.5 N HCl and examined for uroporphyrin. None was found. However, it was observed that the neutralized 0.1 N HCl after extraction with ether continued to fluoresce red in ultraviolet light. This fluorescence was not removed by repeated extraction with ether or ethyl acetate at the prevailing pH, but on adjusting the pH to about 3.5 with HCl, it was found that the porphyrin passed into ethyl acetate. From this it was taken into 1.5 N HCl. The amount of uro-type porphyrin in the aqueous solution was found to represent from 0.15-0.25 μg per 100 grams of brain. Due to various losses in the course of purification the total amount of crystalline material finally obtained was not more than 12 μg. It was esterified and crystallized in the same manner as the coproporphyrin. On account of apparent lipid impurities it was rechromatographed and recrystallized. The crystals were composed of imperfect rosettes of curved hairlike needles. Successive recrystallizations yielded melting points of 272°, 272-3° and 273-4°. The absorption spectrum of the methyl ester in chloroform, using a Zeiss grating comparison spectrometer gave a band in the red, maximal at 626.1 mμ. This agrees well with a uroporphyrin(8). The absorption curve of this porphyrin showed a Soret band maximal at 404.5 mμ for uroporphyrin I. Unfortunately there was insufficient material for decarboxylation and analysis of the isomer type.

Protoporphyrin considerably in excess of coproporphyrin was present in the brain. This in agreement with other observations both in human and animal brain.

It was theoretically possible but unlikely that the porphyrins obtained were derived from the blood contained in the intracerebral blood vessels. Accordingly 50 ml. of beef blood were extracted for coproporphyrin and protoporphyrin(6). A total for the former of 0.72 μg per 100 ml of cells and serum was obtained while for protoporphyrin the value was 16.3 μg per 100 ml. It was clearly impossible that sufficient blood could have been present in the washed brain to account for the amounts of porphyrins obtained.

The isolation and identification of coproporphyrin III confirms the previous tentative results(3,4). The question of significance has been discussed elsewhere(9). The solubility characteristics and melting point of the second compound clearly identify it as a uro-type porphyrin. The melting point of 273-4° C is significantly lower than that of uroporphyrin I methyl ester, 284-6°C(8) yet certain samples of the latter have been noted to melt either lower or higher than this, for reasons that are not clear(8). The slight but significant differences in the Soret bands together with the lower melting point suggest that this may be a Waldenström type porphyrin such as encountered in cases of porphyria, especially in the "cutanea tarda" or "mixed" type of hepatic porphyria(10). This porphyrin incorporates a major portion of uroporphyrin I with a minor fraction of a type III porphyrin having less than 8 COOH groups. The nature of the present porphyrin cannot be determined until larger amounts are available to permit decarboxylation and other studies. Nor is any speculation warranted at the present time as to its biological significance.

Summary. The isolation from beef brain of crystalline coproporphyrin III and of a uro-type porphyrin is described. The ester melting point of the latter is 273-4°C, significantly lower than that of uroporphyrin I methyl ester. The Soret band was also found to differ significantly.

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Phenylpyruvic Oligophrenia Deficiency of Phenylalanine-Oxidizing System. (20163)

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The biochemical nature of phenylpyruvic oligophrenia (phenylketonuria), a disease characterized by mental deficiency and urinary excretion of phenylpyruvic acid, is still uncertain(1). Some indirect evidence was presented in a previous publication(2) which suggests that the metabolic error consists of the inability of phenylketonuric patients to oxidize phenylalanine to tyrosine. Following the recent report of Udenfriend and Cooper (3) demonstrating that an oxidizing system is present in the liver which is capable of catalyzing the conversion of phenylalanine to tyrosine, it became of interest to investigate whether this enzymatic system is defective in phenylketonuria.

Materials and methods. As a source of the enzyme, fragments of liver obtained at the autopsy of 2 phenylketonurics and 3 normal controls were used. The first patient, V.N., a male 25 years of age, died of pulmonary tuberculosis. The liver showed histologically a moderate amount of fatty infiltration. The second patient, W.J., a male 5 years of age, died of acute bronchopneumonia. The liver was histologically normal. In both patients abnormally high amounts of phenylpyruvic acid and phenylalanine were repeatedly found in the urine and the blood. Control 1 was a male, 52 years of age, who died of gastric hemorrhage originating from carcinomatous ulcer; the liver showed a moderate amount of fatty infiltration. Control 2 was a woman, 60 years of age, who died of mitral stenosis. Control 3 was a girl, 8 years of age, whose cause of death was hydrocephalus. The bodies were placed in the ice box (0°-4°C) within a half hour after death. Autopsy was performed 4 hours after death in patient V.N., 5 hours in patient W.J., and 5, 10, and 15 hours respectively in the 3 controls. Fragments of liver were removed, immediately homogenized in chilled Waring blendor with twice their weight of ice cold isotonic potassium chloride and placed in the deep freeze. Two days later the thawed material, packed in ice, was centrifuged for one hour at 8000 r.p.m. and the supernatant fluid, after removal of fatty material, was immediately used as crude liver extract in the test system of Udenfriend and Cooper(3). This consisted of .5 ml of liver extract, 2 μM L-phenylalanine in .20 ml of water, .5 μM of tridiphosphopyridine nucleotide in .20 ml of water, 5 μM of nicotinamide in .20 ml of water and .20 ml of phosphate buffer (pH 6.7). All preparations were commercial products and were not tested for purity. The mixture was shaken in air at 25°C and at the end of one hour 2.70 ml of water was added followed by 1.0 ml of 30% solution of trichloracetic acid. After centrifugation and filtration, tyrosine was determined on 2 ml of filtrate using Udenfriend and Cooper's method(4). The color was read in a Coleman junior spectrophotometer at 450 mμ against a blank consisting of .5 ml of the liver extract treated exactly in the same way but omitting the phenylalanine substrate and the pyridine nucleotide. All tests were run in duplicate.

Results. In Table I the results are presented. It may be seen that tyrosine was formed under the conditions of the experiment in all controls but no formation of tyrosine was observed when liver extracts of phenylketonuric patients were used.

Comment. One may object to the use of autopsy material in the present experiments

TABLE I. Conversion of Phenylalanine to Tyrosine in 2 Phenylketonurics and 3 Controls (2 M L phenylalanine added to each).

		Tyrosine formed, μM
Patient	VN	0
	WJ	0
Control	I	.033
	II	.027
	III	.020

because of the instability of the phenylalanine-oxidizing liver enzyme(3). It should be noted, however, that the controls gave positive results, although the livers were removed somewhat later than in the phenylketonuric patients. Moreover, the amount of tyrosine formed in Control 1 was comparable to that reported by Udenfriend and Cooper(3) in an experiment with rat liver slices in which the liver was immediately removed and all operations carried out in a cold room. In order to avoid further deterioration of the enzymatic activity, no attempt was made at dialyzing the liver preparation and only the crude extract was used.

Although there is a remote possibility that the observed failure to find tyrosine in the phenylketonuric preparations might be the result of a very rapid deamination of phenylalanine to phenylpyruvic acid, the most likely explanation is that the specific enzymatic system described by Udenfriend and Cooper(3) is absent in phenylketonuria. This absence is apparently the essential metabolic characteristic of the disease; in fact, as previously noted(5), the main biochemical findings may be explained on the assumption of a failure in the normally occurring conversion of phenylalanine to tyrosine. Furthermore, the demonstration of the absence of a specific enzyme clarifies the observation that phenylketonuria is determined by a single recessive gene(1), since several instances are on record of specific enzymatic reactions being controlled by single genes.

Summary. Liver extracts of two phenylketonuric patients failed to catalyze the conversion of phenylalanine to tyrosine in the presence of oxygen, phosphopyridine nucleotide and nicotinamide.

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Cross-Dialysis: Description of a Possible Method of Temporary Kidney Substitution. (20164)

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(With the assistance of Mary Z. Shumpert.)

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The several types of artificial kidneys and their clinical applications have been adequately described(1). Basically they consist of two compartments separated by a dialyzing membrane. Blood of the patient is circulated through one compartment and a bath solution is circulated through the other. The dialyzable substances tend to pass from the compartment in which their concentrations are higher into that compartment in which their concentrations are lower. The rate of transfer depends to a great extent on the physical characteristics of the dialyzable substances; the type, thickness and area of the dialyzing membrane; and the concentration gradient present.

The purpose of this study was to design a system of cross-dialysis whereby the blood of a uremic subject would be cross-dialyzed with that of a subject with normal kidneys. There would be no mixing of blood between the two circulations. In such a system it is anticipated that the normal subject would remove through dialysis "toxic products" from the blood of the uremic subject and then utilize its own kidneys to excrete them. This system would not be an artificial kidney in the true sense as it would use a normal pair of kidneys to regulate certain functions which the patient with diseased kidneys would be unable to do. The following results might be expected: a) Re-

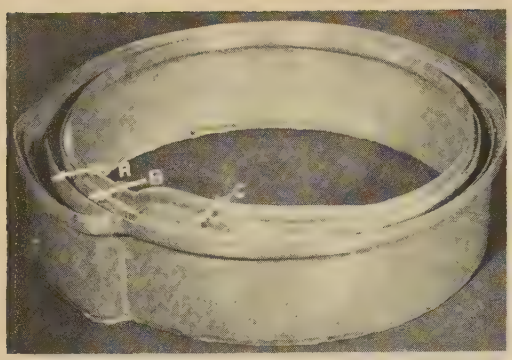

FIG. 1. Dialyzing unit with cover removed. A and C indicate sections where ends of cellophane tubing are brought out for connections. B indicates circular dialyzing compartment which measures 14″ in diameter, 3⅛″ in height and 5/16″ in width.

moval and excretion by the normal subject of such products of metabolism as non-protein nitrogen compounds and possible unknown toxic products which might play a role in the uremic syndrome. b) Automatic control of electrolytes. Those in abnormally low concentration in the patient would be corrected by inflow from the normal subject, and those in abnormally high concentration in the patient would dialyze out and be excreted. c) Similar control as in (b) but for elements and compounds not measurable by present-day laboratory procedures or for which the clinical significance is not yet known.

Method. To accomplish these aims the following apparatus has been devised and constructed. The dialyzing unit, made of stainless steel (Fig. 1) is circular in shape, measuring 14″ in diameter and 3-5/8″ in height. The width of the dialyzing section is 5/16″. Two lengths of cellophane* tubing, measuring 2-7/8″ diameter flat, are placed against each other and are then wound as one for the required number of turns into the hollow section of the unit. When this procedure is carried out, it will be found that every other turn of cellophane represents the same circuit, thereby bringing the dialyzing surface of one circuit directly in contact with the other. The two ends of each length of cellophane tubing are brought out in sections A and C respectively (Fig. 1) where connections are made to 3/8″ diameter polyethylene tubing which is used to bring the subjects' blood to and from the unit. The stainless steel cover, which is not water tight, is applied. Its primary purpose is to help keep the cellophane tubing in place. The present units were used with 8 turns of cellophane tubing representing approximately 5,600 cm^2 of opposing dialyzing surfaces for each circuit. Turns 1, 3, 5 and 7 represent one circuit and turns 2, 4, 6 and 8 represent the other circuit. After assembling, the unit is washed and autoclaved. Immediately prior to operation, it is rinsed with sterile saline and primed with approximately 175 cc of citrated blood per circuit. In the experimental animals, blood from an abnormal subject (uremic) is taken from the femoral artery via a plastic catheter. Its movement is facilitated by the use of a roller-type pump which controls the flow of blood through both circuits. The blood then passes to the dialyzing chamber which is suspended in a water bath kept at 39°C. It flows through the cellophane tubing which is kept compressed, almost flat, by the surrounding tubing and the walls of the unit to give maximum surface area with minimum volume. No shifting of tubing during operation has been noted with the present units. When the blood of the uremic subject is brought into close contact (separated by permeable membranes) with the blood of a normal subject, dialysis takes place. After leaving the dialyzing chamber the blood then flows through a bubble trap and flow indicator and back into the femoral vein of the uremic subject. The blood circuit from the normal subject follows a similar course. Nembutal is used for anesthesia, and clotting is prevented by the use of heparin.

Results. In vitro studies were done with the above apparatus, using 12 liters of a solution containing urea, sodium chloride and potassium chloride in concentrations indicated in Fig. 2. This solution was dialyzed against tap water. There were approximately 6700 cm^2 of opposing dialyzable surface per circuit with a rate flow of approximately 90 cc per min per circuit. Specimens for analysis were taken at intervals from the solution being dialyzed. Results are shown in Fig. 2. As expected, the rate of dialysis decreased as the concentration gradient became smaller. This

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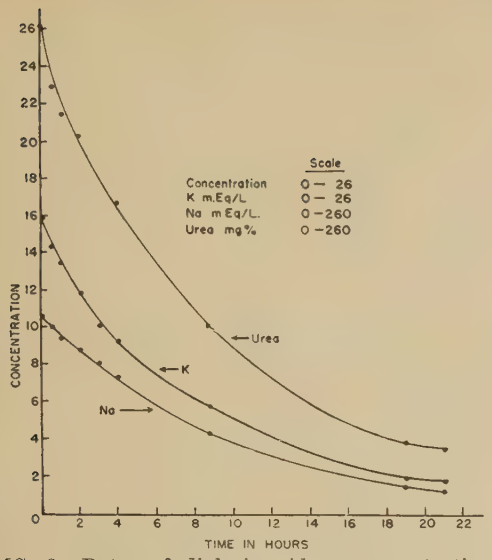

FIG. 2. Rates of dialysis. Above concentrations contained in 12 liters of solution.

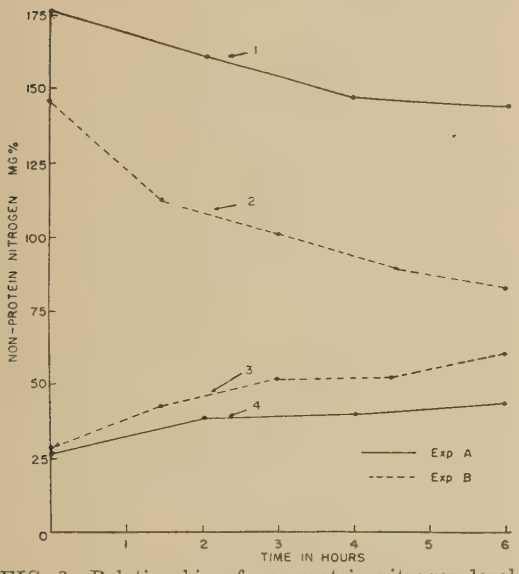

FIG. 3. Relationship of non-protein nitrogen level to hours of dialysis.

experiment was repeated three times with similar results.

In vivo studies were done using dogs as the experimental animals. At first, 2 normal dogs were dialyzed against each other for approximately 8 hours. The apparatus had no ill effects on the animals. Then dogs were made uremic by ligation and division of both ureters. Approximately 48 hours after ligation, the uremic dogs were dialyzed against normal dogs for 6 hours. Fig. 3 shows non-protein nitrogen levels plotted against hours of dialysis. Curves 1 and 2 represent animals with ureters ligated and divided approximately 48 hours before dialysis was begun. Curves 3 and 4 represent normal dogs. In Exp. A one unit was used with 4 turns of cellophane tubing per dog representing approximately 5600 cm^2 of opposing dialyzable surface per dog. The rate of flow was approximately 80 cc per min per dog. Exp. B represents 2 units similar to the above connected in parallel giving approximately 11200 cm^2 of opposing dialyzable surface per dog. The rate of flow for this run was approximately 180 cc per min per dog.

The series is small, consisting of 5 experiments with uremic animals dialyzed against normal animals. In every case, the normal animals survived without apparent deleterious effects. There was no apparent hemolysis. The uremic animals subjected to the above procedure survived approximately 3 days after time of dialysis, a total of 5 days from the time the ureters were ligated. It is believed that the results in Exp. B, using the area and flow noted, were satisfactory. At 6 hours, the concentration gradient between the two animals was small, there being only 20 mg % of non-protein nitrogen difference between the two circulations. The retention of non-protein nitrogen compounds by the normal dogs as shown by curves 3 and 4 is probably to some extent the result of decreased renal function due to prolonged anesthesia(2,3) during dialysis.

Discussion. The method as described demonstrates that it might be conceivable to dialyze a subject in renal failure against a volunteer with normal kidneys. Moreover, one might consider the possibility of dialyzing a patient against a normal animal. It is questionable whether transmission of antigens would occur. The cellophane membrane used will not pass bacteria, viruses, or large molecules such as proteins. If the tubing in one circuit should rupture, it would be unable to penetrate into the other system because rupture of a membrane is immediately detected by the appearance of blood on the surface of the unit. Also, two simultaneous ruptures would be necessary,

one in each circuit, for blood to pass from one system to the other.

Summary. Apparatus and technic for cross-dialysis is described. It is believed that the procedure is relatively uncomplicated, safe and requires few laboratory checks during operation. Only further extensive studies will determine whether or not it has any value as a clinical tool.

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Conjugation of N-Allylnormorphine by Liver Slices.* (20165)

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The respiratory depression caused by an overdosage of morphine can be abolished by small amounts of N-allylnormorphine† (1,2,3). N-Allylnormorphine and morphine differ only in the alkyl group attached to the nitrogen; morphine has a methyl group at this position, whereas N-allylnormorphine has an allyl group attached to the nitrogen. Previous reports (4) have stated that the phenolic hydroxyl group in morphine is the site primarily responsible for the effect on respiration, but now it is evident that changes on the nitrogen atom are important in this respect.

In man (5) and dogs (6) morphine has been reported excreted in urine in both a free and combined state. A study of the action of liver slices on N-allylnormorphine was undertaken to determine whether it was conjugated in the liver like morphine (7) and whether there might be a significant difference in the rate of conjugation to account for the blocking ratio of one molecule of N-allylnormorphine to sixty-seven molecules of morphine (8).

Methods and results. The procedure of Snell and Snell (9) as used by Bernheim (7) was used for the estimation of morphine. This method probably involves the reactivity of the phenolic and alcoholic hydroxyl groups of morphine, for codeine and dihydromorphinone, in which these groups are altered, do not give the same intensity of color as morphine. N-allylnormorphine is identical to morphine except for the replacement of the methyl group on the nitrogen by an allyl group and would be expected to give the same color intensity with the Snell and Snell reagent. That this is so is shown in Fig. 1. The color developed with the silicomolybdic acid reagent is the same for both morphine and N-allylnormorphine in the concentrations used.

In order to study the rate of conjugation of morphine and N-allylnormorphine, livers from dogs anesthetized with sodium pentobarbital were sliced in the usual way. About 300 mg of tissue (wet weight) was placed in 4 ml of Krebs-Ringer bicarbonate solution with either 1.0 mg of morphine or N-allylnormorphine. The slices were incubated at 38°C with a gas phase of 5% carbon dioxide and 95% oxygen in a Dubnoff shaker. Suitable

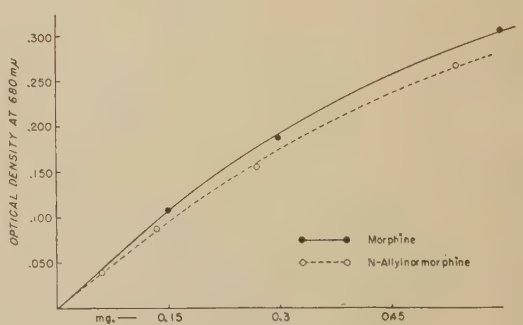

FIG. 1. A comparison of the optical density with the silicomolybdic acid reagent between morphine and N-allylnormorphine.

* This investigation was supported in part by a research grant from the National Institute of Health, Public Health Service.

† N-Allylnormorphine was obtained through the courtesy of Merck & Co., Rahway, N. J.

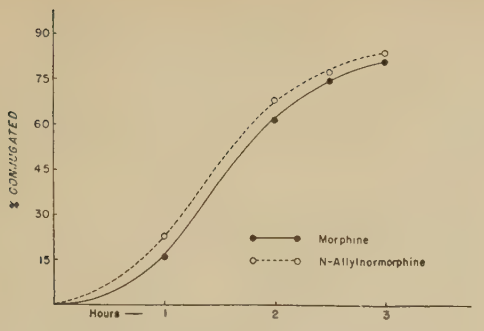

FIG. 2. The rate of conjugation of morphine and N-allylnormorphine by dog liver slices.

controls were incubated at the same time to correct for the effect of the tissue on the silicomolybdic acid reagent. At the end of the incubation time, 1 ml of 20% trichloroacetic acid was added, and after centrifuging, an aliquot was taken for the determination of the unconjugated morphine or N-allylnormorphine.

In Fig. 2 are plotted the results of these experiments. The data for each time period are the average of the results from 3 or more dogs. At the end of two and one-half hours of incubation about 75% of the morphine and N-allylnormorphine is conjugated. While N-allylnormorphine is consistently conjugated more than morphine, the difference is slight and probably not significant.

An aliquot of the solutions was hydrolyzed in 1 N hydrochloric acid by autoclaving for for one-half hour at 20 lb pressure. The average results of free morphine and N-allylnormorphine before and after hydrolysis are shown in Table I. Practically all the free base that has been conjugated can be recovered after hydrolysis.

Discussion. The use of the silicomolybdic acid reagent for the estimation of morphine must not be associated with the nitrogen atom, for N-allylnormorphine gives almost identical color intensities with the reagent. The formation of color by this reagent is therefore dependent mainly on a free phenolic hydroxyl group, for codeine does not give a color with the reagent.

TABLE I. Per Cent Recovery of Alkaloid.

	Autoclaved	
	Before	After
Morphine	35	98
N-Allylnormorphine	33	93

300 mg of tissue incubated 2 hr. Autoclaved in 1 N hydrochloric acid ½ hr at 20 lb pressure.

The conjugate that is formed *in vivo* and *in vitro* with morphine is presumed to be the glucuronide(5) but the conjugate has not been isolated or characterized. The rate of conjugation by surviving dog liver slices is the same for both morphine and N-allylnormorphine, as shown in Fig 2. Probably, the same enzyme system is responsible for the conjugation of N-allylnormorphine as is operating in the conjugation of morphine.

Conclusions. 1. N-Allylnormorphine can be estimated by the use of the Snell and Snell reagent, for a change in the alkyl group on the nitrogen does not change the intensity of the color produced. 2. N-Allylnormorphine is conjugated by surviving liver slices at the same rate as is morphine. In two and one-half hours, about 75% of the two compounds is conjugated. 3. Because conjugation of the two compounds proceeds at essentially the same rate and the effective blocking dose of N-allylnormorphine is small, the latter compound must be more firmly fixed to the receptor site than is morphine.

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Effect of Vaccinia on Cells Grown in Tissue Culture. (20166)

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The cytopathogenic effect of viruses on cells in tissue cultures, as described by Enders, Robbins, and Weller(1) in connection with the poliomyelitis viruses represents a syndrome of pathologic changes in the cultured cells that in its end results can be broken down into the following symptoms: 1) degenerative changes in cell morphology due to the virus; 2) inhibition of cell migration due to the virus; and 3) changes in tissue metabolism as indicated by decreased acid production. These effects can all be observed by inspection of the cultures, as well as by observation of the changes in the gross morphology of the growing fragments under regular microscopy at magnifications ranging from 100 to 400. In the present study the cytopathogenic effect of the vaccinia virus on the cells cultured *in vitro* was followed principally in its manifestation of degenerative changes in the cell morphology beginning with cell injury through complete cell degeneration and destruction. The observations at this point were undertaken without the advantages offered by the use of phase microscopy with its opportunities for a dynamic study of the actual action of the virus on the cell or the use of electron microscopy and micrography.

The experiments presented in this paper were aimed at the establishment of the fact that the cytopathogenic changes caused by the vaccinia virus in the growing tissue *in vitro* were present, that they were definite and specific, and that they were not due to incidental or accidental influences. It is a well known fact to all workers in the field of tissue culture that for obvious reasons the necessary use of substrates and nutrients consisting primarily of so-called natural components present the investigator with an endless array of unknown variables. Extensive studies have been made of the physical, chemical, biochemical and physiological knowns and unknowns which may cause changes in cell morphology, theoretically and actually amounting to cytopathogenic effects. But in virology, where the growing tissue represents the substrate and the nutrient, *i.e.*, the host for the virus, one can only hope to strike an average in supplying the tissue and the virus with ingredients which permit sufficient multiplication for the first and, consequently, for both.

Methods and material. The roller tube experiments constituting the major portion of this study employed the usual technics and procedures, and a detailed description would only be repetitious(2). In several experiments the Maximow double cover slip method (3) was used, which permitted observation of the cultures under high power microscopy. In the roller tubes, the finely minced tissue fragments were planted in a single vertical row of explants at 10 to 15 mm intervals inside the tube on a thin coat of heparinized plasma. Clotting was induced by adding 2 to 3 drops of chick embryo extract. The liquid phase of the medium consisted of 1.5 ml of nutrient, composed of a balanced salt solution, ox serum ultrafiltrate, embryo extract, and horse serum. This liquid phase varied in the percentage composition of the single ingredients, not only for suitability to the different tissues used, but also to the virus. Since an extremely rapid rate of growth is not optional in virus studies, a more or less "lean" liquid phase consisting of 85 to 90% of balanced salt solution containing 1:4 ultrafiltrate of ox serum, 5 to 10% diluted embryo extract, and 5% of horse serum, inactivated at 56°C for 30 minutes was used as warranted. This composition closely follows that recommended by the Enders group(4). The balanced salt solution was prepared according to the formula of Hanks (5). Chick embryo extract and plasma were also prepared in our laboratory. Ox serum ultrafiltrate and horse serum were obtained commercially. The liquid phase also contained phenol red indicator in a final concentration in the medium of 2 mg %, with the final pH of the medium corrected by the addi-

tion of 1.4% isotonic sodium bicarbonate to 7.2. Penicillin was used in amounts resulting in final concentrations in the medium no higher than 100 to 300 units/ml—a concentration known to be well below inhibitory or toxic effect. Chloromycetin was also tried in several experiments in extremely small concentrations without apparent interference with the objectives of the study. The following tissues were used: (a) chick embryos at 10 to 11 days incubation, minced as a whole and fragments picked at random; (b) chick embryo hearts at 12 days incubation; (c) chick embryo skin and muscle from legs and backs of embryos incubated for 12 to 13 days; (d) human embryonic skin and muscle; and (e) human embryonic kidney tissue. With the use of material coming from human and animal sources in studies of virology, there is always the additional hazard of introducing unknown virus from the tissue source into the culture(6). To eliminate this hazard 40 series of experiments were run and the soundness of components and sources was determined before the validity for their use was established.

Virus inoculum. The virus used in our experiments was prepared in two ways. One mode of preparation was to make a 20% suspension in buffered saline of the chorioallantoic membranes of chick embryos of 12-day incubation, inoculated with the 72nd egg passage of the CVI strain of vaccinia. The second preparation consisted of a 20% suspension in saline of the washed elementary bodies obtained from the same egg passage. The chorioallantoic membrane suspension was ultracentrifuged at a speed of 40,000 RPM and the sediment containing the elementary bodies was resuspended and recentrifuged at the same speed and, finally, made up to the original volume.

During the course of the study the identity and infectivity of the virus preparations used was confirmed in various ways: (a) inoculation of the chorioallantoic membrane of 12-day-old chick embryos with the suspensions as used in the tissue culture experiments, and close observation for the appearance of characteristic pock lesions; (b) inoculation of the material on the shaved skin of white rabbits. Titration of the virus content of the suspensions was carried out by hemagglutination tests. Specificity was confirmed by using antiserum from patients proven to have high anti-vaccinial titers in hemagglutination inhibition tests(7). In the Maximow double cover slip experiments the components of the substrates for the tissue culture, the tissues used, and the virus suspensions were the same as used for the roller tube experiments. Here, too, the tissue fragments were embedded in a thin plasma clot, and the feeding solution consisted of 3 drops of liquid composed of ultrafiltrate of serum, serum and diluted embryo-extract. Of all the possible variations available in inoculating tissue cultures with virus, the simplest method was used, *i.e.*, the addition of virus suspensions to the liquid nutrient phase. In the case of the roller tubes containing a liquid nutrient of 1.5 ml volume, the virus suspension consisted of 0.1 to 0.2 ml. In the Maximow slides, one drop of the suspension was added.

Results. (See Table I).

TABLE I. Days after Inoculation of Cultures of the 73rd Egg Passage of CVI Vaccinia Virus in the Form of (a) Washed Elementary Bodies and (b) Chorioallantoic Membrane Suspensions. 15 experiments.

—Vaccinia inoculated cultures—			
Age of cultures at inoculation	Cytopath. effect first noticeable after inoculation, days	Complete degeneration cytopath. effect at its max.	Uninoculated control cultures—day of discard
10	6	12	50
8	6	10	40
6	3	9	19*
6	3	9	19
10	3	14	28
10	5	14	28
4	4	9	24
6	3	6	26
6	3	6	26
6	3	8	26
5	3	6	19
6	4	7	16
6	4	7	16
5	5	8	33
6	3	8	21
Avg 6.6	3.9	8.9	

* Except for Exp. 1 and 2 in the control experiments there was no all over degeneration at conclusion of experiments. Many fragments in the control series still showed healthy growth.

Characteristic degenerative changes comparable to cytopathogenic changes as produced by poliomyelitis and other viruses (1,8,9), first became evident between the third and sixth day after introduction of the virus. It should be noted here that for purposes of standardization in all experiments, the tissue fragments were allowed to grow out to a strong, healthy tangle of fibroblast-like cells, or characteristic sheets of epithelial cells or wide strands of muscle cells, dependent on the material used. No histologic identification of cell material was attempted. Another point kept in mind was that in all experiments only original explants were followed, studies on subcultures will be made at a later date.

The earliest noticeable degenerative change, occurring between the third and sixth day, was the appearance of fatty refractile globules and liquid droplets causing swelling of the cells, amounting—somewhat later—to distortion into a variety of bizarre shapes. It is established that the vaccinia elementary bodies contained in the inclusions observed in the stained preparation of vaccinia infected cells, as well as by electron microscopy, are connected with lipoid material(10). It is possible that the tendency of cells to deposit fat droplets in certain conditions of tissue culture enhances the chance of the virus to invade the cell. As the cells swell and inflate, the continuity of the outgrowing aureoles around the explants is disrupted and the contact between the cells broken. The fatty refractile material in the cells is surrounded by dense granules, and the fusiform cytoplasm of fibroblast-like cells becomes less and less evident. The next stage seems to be a release of the large droplets, complete granulation, balling and rounding of the cells, and finally complete fragmentation and disintegration until only cell debris is left in the field. This process seems to work from the periphery of the radial outgrowth toward the center and the original implant and takes place within 8 to 10 days.

In the first experiment to show definite and striking pathogenic cell degeneration, the virus was introduced in the form of washed elementary bodies 8 days after the start, with complete degeneration 6 days later. In this experiment besides morphological cytopathogenicity, another aspect of the syndrome was evident, *i.e.*, decrease of acid production due to metabolic changes. At the conclusion, the inoculated roller tubes showed a pH average of 7.2, while uninoculated cultures indicated a pH of 6.6. However, it should be pointed out that in roller tubes the pH differences in infected and normal cultures do not follow the course observed in suspended cell cultures(1), since there are additional factors present which may play a part. Healthy, strongly metabolizing tissue fragments in roller tubes often cause rapid liquefaction of the surrounding plasma clot substrate, which cannot be remedied with patching. If this happens the fragment seems to be cut along a tiny sharp line of incision, often crescent-shaped. Consequently, fragment particles and portions of fresh outgrowth drop into the medium and are inadvertently carried off at the next nutrient change, reducing the total amount of tissue present in the tube. In contrast to this, it was frequently observed that infected and degenerated fragments with their outgrowing coronae seemed to be fixed in position in their original rough outlines. Thus, the healthy controls with less tissue did not produce the rapid pH decline, while the infected tubes still continued to show pH changes, even though diminished.

In order to compare the action of washed elementary bodies and chorioallantoic membrane suspension, a number of experiments were run in parallel series—one part of the cultures being inoculated with washed elementary bodies, and the parallel run with the membrane suspension. In all experiments, *i.e.*, roller tubes and Maximow slides, control preparations equal in number to inoculated preparations were always set up in identical procedures, except for the addition of the virus.

Discussion. The vaccinia virus, both in the form of washed suspensions and in the membrane suspension, seemed to affect the tissue cultures and effect cytopathogenic changes. At the risk of pushing a point too far it may be stated that the washed elementary bodies seemed to go to work somewhat more rapidly. The time table of changes effected followed a

close sequence of events and complete degeneration was attained about 10 days after inoculation. At the peak of the changes effected, the contrast between healthy and infected cultures was very striking. As is well known, eventually healthy cultures will also undergo degeneration and necrosis and the gross appearance of these changes may closely resemble the changes called cytopathogenic when referring to infected cultures. However, this normally happens several weeks after injury and destruction of cells in virus infected cultures takes place.

Specificity of virus action was observed in several experiments, where specific antiserum from patients known to have a high antibody titer to vaccinia, was allowed to act on the virus and apparently had a decelerating and, in some cases, completely inhibitory action. This aspect of the study, and the question of survival and quantitation of the virus in supernatant fluids, is being studied at present.

Conclusions. The cytopathogenic effect of vaccinia virus in roller tubes and Maximow double cover slip tissue cultures was established in its principal aspect of degenerative and pathological changes in cell morphology. Specificity of this effect was corroborated as far as possible at the present with coordinated serological tests and animal experiments. Further studies and analysis of visual changes by phase microscopy and photomicrography are planned.

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Eosinophile in Human Skin Homografting.* (20167)

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The revival of interest in skin homografting is evidenced by the increase in the number of papers which have appeared in the medical literature within the past several years. Many of these reports explain the sloughing of most skin homografts on the basis of an *acquired immunity reaction.* In reviewing the literature(1-4) it becomes apparent that skin homograftings have been permanently successful only between identical twins(5), or between members of the same family(6,7). That the compatibility of blood types between donor and recipient remains an important factor necessary for permanent survival of homografts is indicated by other recent reports (4,8,9). Extensive skin homograft research in animals has been described(10-15). Brown and McDowell(16) found "many eosinophiles" in biopsies taken of the sloughing homograft in split-thickness homografting. Split-thickness homografting in a child re-

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ceiving ACTH therapy was reported by McNichol(17); the eosinophile count began to rise prior to and during the breakdown of the homograft despite the depression of the total eosinophile count by ACTH therapy early in the immediate post-operative course. Animal experiments have demonstrated similar findings. Rise in the eosinophile count at the time of homograft sloughing in rabbits, despite depression of the eosinophile by ACTH and desensitization procedures has been described (18).

Our interest in the eosinophile response was stimulated by observations of a split-thickness homograft from a white donor placed in a forehead defect of a Negro. Microscopic examination of biopsies taken at the time of the sloughing of this *first-set* homograft disclosed eosinophilic engorgement under the homograft dermis. The eosinophilia, in this case, differed from the classical reports(19) of an increased number of lymphocytes in the region of a sloughing skin homograft. A rise in *circulatory* eosinophiles was also noted in our patient at the time of a *second-set* homografting with skin taken from the same donor. On the basis of these observations, a study was made of the circulating eosinophiles and of tissue eosinophiles in ten cases of full-thickness skin homografting.

Experimental homografts in man. Full-thickness homografts of identical size were placed in similar locations (anterior thigh) in surgically created defects and in two cases, full-thickness autografts of identical dimensions were employed on the opposite thigh. All autografts took initially and survived as permanent grafts. The subcutaneous fat was trimmed away from the under surface of the dermis in both homografts and autografts, and the grafts were secured in position by similar types of dressings. *Full-thickness* homografts were used as a matter of expediency. It was simpler to remove a uniform-sized, round piece of full-thickness skin and to fill the resultant defect with a full-thickness homograft of equal size. The uniform size employed served to minimize variations in the eosinophile response which might be attributed to the dosage-phenomenon(11).

Circulatory eosinophilia. Daily white cell counts and differential counts were made in each homografted patient, the total number of circulating eosinophiles being determined by multiplying the white count by the number of eosinophiles in the differential count; this eosinophile count was found comparable to that obtained in total eosinophile counts determined by the Dunger chamber technic. The white counts, differential counts and total eosinophile counts in these patients were taken at various times of the day; in the majority of instances in the mid-morning, about 2 hours after breakfast. The effect of meals on the eosinophile count can be disregarded (20). The normal eosinophile count in ungrafted, control patients is 80-500 cells per cubic mm. The highest eosinophile counts obtained in each of 10 homograftings (4 of these were second-set homograftings), were 4,301; 4,080; 3,047; 1,374; 1,138; 1,033; 975; 800; 540; and 484 cells per cubic mm. The highest eosinophile counts obtained in each of 4 additional autograftings which served as controls were 960; 868; 639; and 552 cells per cubic mm. Penicillin was employed in all of our cases. In 6 of the 10 homograftings, the counts were more than twice the normal and in two were 8 times normal; one was 6 times normal and another 3 times normal; in none of the autograftings did the count exceed twice the normal range. In each case of homografting, however, the rise in the number of circulating eosinophiles exceeded twice the normal range (Fig. 1). Following disappearance of the rejected homografts, the eosinophile counts fell to pre-operative levels. A single first-set homograft and a single second-set homograft from the same donor may provoke a highly specific and similar response from the host. This is suggested by the graphs of the eosinophile levels of the first- and second-set homografts (Fig. 1 and 2).

In one patient, can it be considered mere coincidence in both sets of homografts (Fig. 2) that the eosinophilia charted for each set so closely paralleled each other, or that the return of the eosinophile count to normal occurred on the very same day (34th post-operative day) in both sets, and further, that a secondary eosinophilia with a similar but less

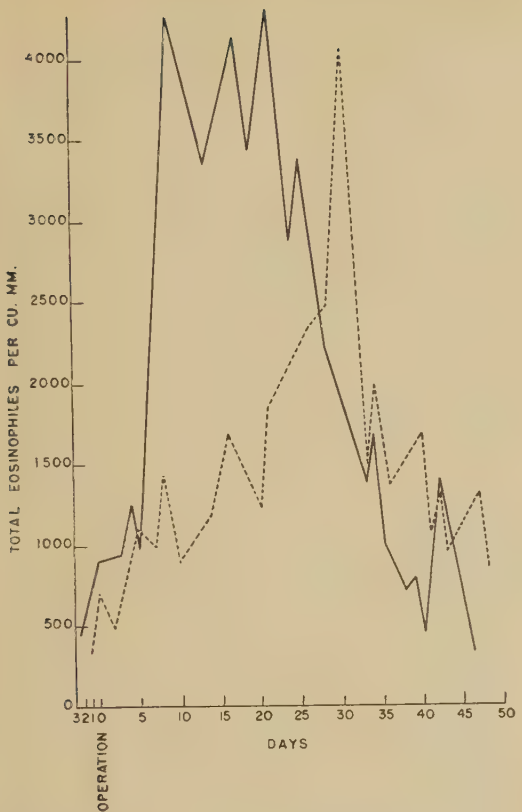

FIG. 1. Case A—The eosinophilia represented by black line for the first-set homografting, and by a broken line for the second-set. Note that the intensity of eosinophilia for both sets exceeds 4000 cells per mm^3. These lines fall back to normal levels only when the last vestiges of the homograft dermal pads have disappeared.

intense peak of activity followed the primary eosinophilia? Is a significant factor of homografting, as yet unexplained, involved in this occurrence?

No similar increase in eosinophiles could be demonstrated in the 4 autografting, nor was eosinophilia demonstrable in an additional case in which a refrigerated *split-thickness* Negro homograft failed to take initially on its white recipient. The graft was found to have sloughed at the first change of dressing on the 5th post-operative day. The failure to "take" in this case was attributed by us to the fact that the method of refrigeration employed had affected the graft and that a devitalized graft had been applied. If a devitalized graft fails to elicit an eosinophile reaction, is the eosinophilia then a response only to "living" homograft cells? Swanson *et al.* (20) found that diurnal variation of the eosinophile count resulted in a greater *eosinopenia* in the morning hours. The findings of morning *eosinophilia* in the homografted patients of this study, therefore, seem to be more striking when compared to the eosinopenia in control patients; the eosinophilia is also in marked contrast to the eosinopenia noted in the post-operative courses of patients who have undergone major surgery (21-23).

Tissue eosinophilia. Circulatory eosinophilia in skin homograftings was accompanied by an increase in *tissue* eosinophiles in the region of the sloughing homograft; the eosinophile count in the tissues was determined by the following method: Freshly removed biopsy specimens consisting of equal halves of homograft and of marginal skin of the host were immediately immersed in Bouin's fixative, infiltrated and embedded in paraffin, sectioned at 8 micra and stained with hematoxylin and eosin. Magnification with an oil immersion objective (x95) and a No. 10 ocular lens was used to count the eosinophiles in one field (x950), the slide was moved one field diameter, and the number of eosinophiles again recorded; fifty adjacent fields were thus counted in two separate parts of each biopsy section. One of the areas included the junction of the host and graft tissue; the other consisted of the graft tissue. Counts were made on 4 alternate sections from each biopsy specimen; the total number of eosinophiles in the 400 oil immersion fields was recorded. The tissue eosinophilia was found comparable in magnitude to the intensity of the circulatory eosinophilia. This would seem to support the impression that the eosinophile response is directed against the presence of a homograft.

Interpretation of findings. a) The greatest intensity of eosinophilia occurred when the epithelium of the homograft had sloughed and only the dermis remained intact. b) The eosinophilia subsided and the count returned to normal levels as the last vestiges of the homograft's dermal pad sloughed away. c) The number of eosinophiles in a differential count increased at the expense of the polymorphonuclear cells, thus establishing an in-

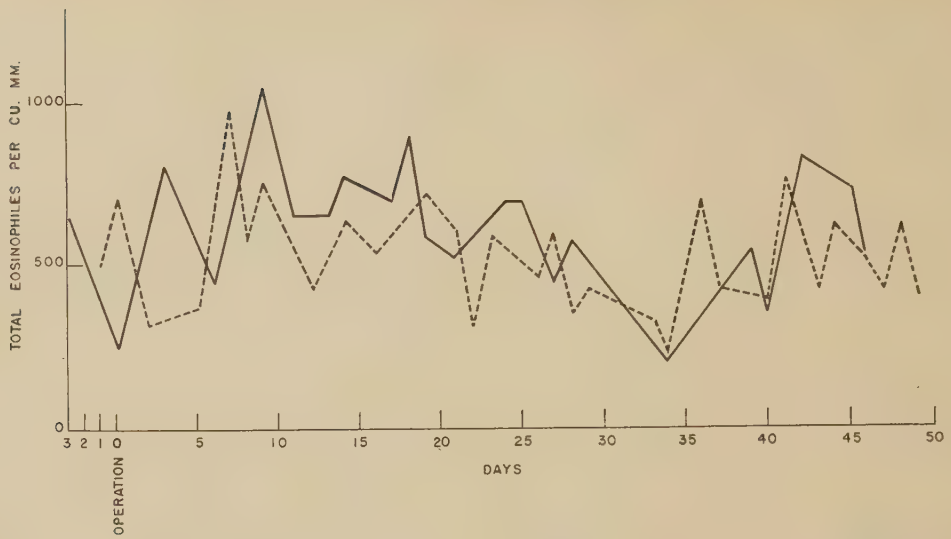

FIG. 2. Case B—Close parallelism between eosinophilia charted for first-set homografting (black line) and that charted for second-set homografting (broken line). In both cases the lines dropped to an almost identical number of eosinophiles per mm³ on the very day (34th post-operative day) when the last vestiges of the homograft dermal pads have disappeared.

verse relationship between the two types of cells in their responses to homografting.

Summary and conclusions. 1. In full-thickness homografts in man, an increase of eosinophiles occurred in the circulating blood stream and in tissues at the site of the graft. 2. Tissue eosinophilia is comparable in magnitude to the intensity of the circulatory eosinophilia. 3. Eosinophilia seems to be most intense when the epithelium of the homograft has sloughed and only the dermis remains intact. 4. Eosinophilia subsides and the count returns to normal levels as the last vestiges of the homograft's dermal pad sloughs away. 5. The number of eosinophiles in a differential count increases at the expense of the polymorphonuclear cells, thus establishing an inverse relationship between the two types of cells in their responses to homografting.

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Bacteriology of the Healthy Experimental Animal. (20168)

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The bacterial flora normally resident in the tissues of the abdominal viscera vary considerably among different species. In appropriate circumstances, the interpretation of experimental data should take account of the varying incidence and variety of such resident bacterial flora. For this reason, the bacteriology of the organs and tissues of normal dogs, rabbits, guinea pigs, rats and hamsters was studied and is herewith reported.

Technic. Healthy animals, resident for at least one week in a clean, air-conditioned animal farm, were killed by intramuscular nembutal or ether and autopsied immediately after death. Tissue specimens were taken as follows: The skin was shaved, washed with aqueous zephiran, dried and then burned with a gas flame until carbonized. The burned surface was removed with sterile instruments and a piece of the skin beneath excised for culture. Connective tissue, fascia and muscle were burned and treated similarly. The thoracic and peritoneal cavities were then opened with sterile instruments. All organs to be cultured were flamed *in situ* and cubes of tissue, ½-1 cm in length, were removed, flamed briefly, streaked with some pressure over Endo and blood plates and then plunged into 10 cc of thioglycollate (Brewer) covered with paraffin. The blood plates were incubated in a Brewer jar. Blood, bile, and urine were drawn with sterile syringes after flaming the vessel wall, and cultured similarly. Tubes and plates were observed up to 96 hours, if no growth occurred earlier. Aerobic organisms found were identified by routine bacteriological methods. When smears of growth in thioglycollate showed large gram-positive bacilli, aerobic subcultures on agar and blood plates were made to exclude aerobic bacilli of the subtilis group. These large gram-positive bacilli always proved to be anaerobic. Fifty of these strains were identified by comparison of the detailed test data against those of anaerobic strains classified in Bergey's Manual. One hundred and twenty additional strains of clostridia were recovered and were tentatively classified by responses to the following tests: hanging drop, gram and capsule stains, blood plate, litmus milk, and sugar fermentations. All seemed to belong to the first group described below. The high frequency of clostridia in the dog's tissues led to repeated checking of instruments, test tubes, culture media, etc. These were always found sterile.

Results. Incidence of Bacteria in Different Species. Dogs. See Table I. The aerobic bacteria found were always gram-negative bacilli. They were found only rarely (4% of 228 cultures) and only in subcultures from thioglycollate in very small numbers. Of the 9 positive cultures, 8 were *E. coli*, 1 was *Pseudomonas*. Smears from some 50 other thioglycollate cultures showed gram-negative bacilli in addition to large gram-positive bacilli. But since aerobic subcultures of these were sterile, the gram-negative bacilli may be considered non-viable, or as belonging to the genus *Bacteroides* a normal inhabitant of the intestines. No attempt has been made to identify this species. Of the 228 cultures, 61% were positive for anaerobic, gram-positive spore bearers. If we exclude all cultures of body fluids, which were uniformly sterile, this percentage is 80. *Rabbits.* In 159 cultures from rabbits, 29% of all anaerobic cultures were positive for clostridia, but 40% of cultures from tissues were positive for anaerobic spore-bearers. Only 1 positive aerobic culture was obtained. This was from a liver and showed *E. coli* and *A. aerogenes*. *Rats, Guinea Pigs, and Hamsters.* 75 cultures from 15 rats, 60 cultures from 10 guinea pigs, and 70 cultures from 10 golden hamsters, both aerobic and anaerobic, were all sterile (Table I).

Comment. Positive clostridial cultures were obtained only from subcultured thioglycollate, never from direct anaerobic blood plates. Hence their numbers in the tissues cultured must have been very small. They were never

TABLE I. Clostridia Recovered from Normal Organs.

Organs	Normal dogs		Normal rabbits		Normal hamsters		Normal guinea pigs		Normal rats	
	No. of spec.	% pos. cultures	No. of spec.	% pos. cultures	No. of spec.	% pos. cultures	No. of spec.	% pos. cultures	No. of spec.	% pos. cultures
Skin	10	80	6	67	10	0	—	—	—	—
Muscle	17	76	13	23	10	0	10	0	15	0
Heart muscle	16	81	6	50	10	0	—	—	—	—
Portal vein	12	17	11	9	—	—	—	—	—	—
Vena cava	12	0	9	0	—	—	—	—	—	—
Lung	20	80	11	45	—	—	—	—	—	—
Pancreas	15	87	15	46	10	0	10	0	15	0
Liver	20	90	15	46	10	0	10	0	15	0
Kidney	16	75	15	20	10	0	10	0	15	0
Urine	6	0	5	0	—	—	—	—	—	—
Adrenal	12	75	7	57	—	—	—	—	—	—
Thyroid	13	77	5	20	—	—	—	—	—	—
Spleen	18	83	13	39	10	0	10	0	—	—
Testis ovary	10	80	6	33	—	—	—	—	—	—
Bile	12	0	7	0	—	—	—	—	—	—
Peritoneum	19	16	15	0	—	—	10	0	15	0

TABLE II. Incidence of Clostridia by Sampling Similar Areas of Different Livers.

Liver	Right lobe near hilus	Right lobe near periphery	Right middle lobe	Left lobe	Quadrangular lobe	Three adjacent samples
1	Many clostridia	S*	S	S	S	Many " "
2	Sterile	Few	M	Many	M	" Few M
3	Sterile	Many	S	M	S	Many Few "
4	Few	M	Many	Few	Few	Many M Few

* S = sterile; M = moderate.

present in bile, urine or vena cava blood. Rarely they were found in peritoneum and in portal blood. A sterile culture from a small sample of an organ does not exclude the presence of bacteria in the entire organ. Table II shows that there is a very irregular distribution of clostridia in the dog's liver. Hence the figures underestimate contamination for large organs which cannot be cultured *in toto*.

Classification of Positive Cultures. Fifty strains of the clostridia isolated could be divided into 3 groups on the basis of a detailed morphological and cultural analysis. The first group of 30 strains showed all the morphological and cultural characteristics of *Cl. welchii* (*perfringens*) as described in Bergey's Manual of Determinative Bacteriology (1948). A second group of 12 strains showed the following characteristics: Clotting of litmus milk delayed (48-96 hours); acid and gas in dextrose, but not in lactose or sucrose; weak or negative hemolysis on anaerobic blood plates. Other morphological and cultural findings were identical with those of group 1. The third group of 8 strains showed the following: Litmus milk unchanged or very slightly acidified, but no clotting after 2 weeks. Dextrose unchanged or slightly acid without gas formation; other sugars not attacked. Liquefaction of gelatin slow. No hemolysis on anaerobic blood plates. H_2S not produced. Other findings similar to group 1.

It was not possible to identify the strains of the second and third groups from the list

in Bergey's Manual (1948). This Manual lists among 60 species of clostridia (excluding those listed in appendix I and II), 8 species which are not motile. Of these only 3 are capsulated (*Cl. welchii, Cl. malenominatum, Cl. angulosum*). Groups 2 and 3 as described above do not show the cultural characteristics of any of the 3 species. Group 3 has some resemblance to *Cl. malenominatum* (no coagulation of milk, lack of sugar fermentation), but this species has been isolated only once from the feces of a diarrheal infant and is said to be very pathogenic to guinea pigs, a property not found in Group 3 strains. Nor are Group 2 or 3 identifiable as the bacillus which Wolbach and Saiki(1) cultured from the normal dog's liver.

Toxicity of the Clostridia. Identification of a strain of *Clostridium* as *Cl. welchii* (*perfringens*) requires the demonstration that it is able to produce a soluble toxin. The ability of these strains to produce exotoxin was investigated as follows. Cultures in thioglycollate or pancreatic digest of beef heart(2) were incubated 2-7 days and filtered through Seitz filters. Filtrates were tested for sterility and then injected in volumes varying from 1-5 cc subcutaneously in one group of guinea pigs, and intramuscularly in a second group. In spite of such large doses, none of the animals died or showed any local reaction at the point of injection up to a period of 3 weeks. Hence the strains examined did not produce exotoxin in the test tube in any demonstrable amount. [Injection of filtrates into the breast muscle of pigeons, as suggested by Logan(2) for demonstration of minimal amounts of exotoxin, has not been done.] But results were different when pure cultures of eight strains, grown in thioglycollate for 3-5 days, were injected intraperitoneally or subcutaneously into healthy guinea pigs. Sterile thioglycollate was injected in similar volumes into other guinea pigs serving as controls. Four of eight animals injected intraperitoneally with 5 cc of the thioglycollate culture died within 6-24 hours; the other 4 survived. Two injected with 4 cc and 3 with 3 cc all survived. The dead guinea pigs showed hyperemia of the peritoneum with a very small volume of bloody fluid from which the injected strain was recovered in pure culture. In 2 of them liver cultures were done and revealed the injected clostridia in pure culture, while normal guinea pig livers always proved sterile.

Of 8 guinea pigs injected subcutaneously with 5 cc of the same cultures, 6 died within 6-24 hours and 2 survived. Of 8 injected with 3 cc two died. Postmortem showed widespread hemorrhagic edema of the subcutaneous tissue. The peritoneum was hyperemic. Cultures of the subcutaneous tissue, peritoneum, and liver revealed pure cultures of the strains injected. No gas bubbles were found even when the autopsy was done a few hours after death. Only guinea pigs injected with strains of the first group, *i.e.*, probably *Cl. welchii*, died. Control guinea pigs for each group, injected with sterile thioglycollate, were unaffected by the injection.

Additional evidence that the clostridia from normal dogs' livers are *Cl. welchii* is the following: An aseptically prepared homogenate of dog's liver was injected intraperitoneally into normal guinea pigs and into guinea pigs which had received 3 cc of polyvalent clostridial antitoxin* subcutaneously daily for 3 days prior to injection. Many more untreated than treated guinea pigs died (Table III). Since the cultures of the strains recovered correspond to those of *Cl. welchii* and to none of the other species, it is clear that the antitoxin protected against *Cl. welchii* and that these bacteria produced exotoxin.

Frequent transfers of cultures in the thioglycollate or cooked meat medium did not encourage toxin production, nor did the strains recovered from dead guinea pigs show any increased toxicity.

Discussion. Earlier bacteriologic studies of normal tissues, beginning in 1885(3,4,5,6) showed no bacteria in the organs of rabbits or guinea pigs, or the mesenteric lymph glands of cattle. The cultures in these studies were observed for only 3 days. Ford(7) found bacteria in rabbit, guinea pig, dog and cat tissue cultures after 5-17 days. But these bacteria were non-hemolytic *Micrococcus pyogenes*, var. aureus and albus, *Bacillus mesen-*

* 15 cc of this polyvalent antitoxin (Lederle) contains antibodies against *Cl. welchii* (10,000 units), *Cl. septicum* (10,000 units) *Cl. novyi* (1500 units), *Cl. bifermentans* (1500 units), *Cl. histolyticum* (3000 units).

TABLE III. Intraperitoneal Injection of Homogenized Dog Liver into Guinea Pigs.

Vol. inj., cc	No antitoxin		Antitoxin	
	No. of animals	Death, %	No. of animals	Death, %
7	4	75	4	25
6	4	100	4	25
5	14	57	12	0
4	14	21	10	0
3	14	30	10	0
Summary	48	46	40	5

tericus, Bacillus cereus, var. mycoides, *Bacillus megatherium,* and *Proteus, i.e.,* non-pathogenic bacteria which were very likely contaminants. Gage(8) found bacteria in nearly all livers of healthy dogs, but did not identify the species. Wolbach and Saiki(1) found clostridia regularly in the livers of healthy dogs, but considered them not to be *Cl. welchii.* Others confirmed these findings(9,10,11). Dragsted *et al.*(12) considered the clostridia which they found regularly in the dog's pancreas to be *Cl. welchii.*

It appears that all organs of the healthy dog, not only liver and pancreas, are more or less regularly contaminated with clostridia. These are presumably invaders from the intestine. That they invade *via* the portal vein is suggested by the finding of clostridia in the portal vein in 2 of 12 specimens, while their incidence in 12 specimens of vena caval blood was zero.

Presumably, the bacteria in the tissues of normal animals are located intracellularly, because the secretions of organs containing clostridia were always sterile. Since absorbable intraintestinal antibiotics can eliminate clostridia from the intestine, but not from the liver of the healthy animal, those in the liver of the healthy animal are not in a stage of multiplication. But the protection afforded by active and passive immunization against clostridial exotoxins and by penicillin and aureomycin in certain experimental conditions which activate these bacteria(15,16) demonstrates the need to consider carefully the role of these bacteria in experiments involving the abdominal viscera of dogs and rabbits.

Two observations of interest are the following: 1) Fifteen liver biopsies from humans taken during operation were sterile for aerobes and anaerobes, thus confirming the findings of Romieu and Brunschwig(13) and Sborov *et al.*(14); 2) The organs of 10 nearly mature dog embryos from 2 mother dogs, which showed the usual contamination, were sterile.

Conclusions. 1) The organs of normal rats, guinea pigs and golden hamsters are sterile. Blood from vena cava, bile and urine of normal dogs and rabbits was always sterile. Most of the tissues of healthy dogs, and rabbits to a lesser degree, harbor clostridia. The embryos of healthy dogs are sterile. 2) The great majority of the strains recovered from the tissues of dogs and rabbits show all the morphological and cultural characteristics of *Cl. welchii* (*perfringens*). Toxin production, though weak, was demonstrated.

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Comparison of Respiratory Enzyme Levels in Tissues of Mammals of Different Sizes. (20169)

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Various structural and functional features of an organism are known to vary with body size(1). The decrease in respiratory metabolic intensity with increase in body size is reflected in a decrease of the respiratory intensity of several homologous tissues(2).

There is then an inverse correlation of both total and tissue metabolic intensity and body size among different species, although intraspecific comparisons do not always demonstrate this relationship(3). Since there appear to be differences in the *in vitro* respiration of the homologous tissues of mammals of different sizes, it would appear that the next logical step would involve analysis of the intrinsic factors involved in cellular respiration to determine whether they too will demonstrate an inverse relationship with body size. Therefore succinoxidase and malic dehydrogenase activity in the tissues of the mature cow, rat, and mouse was measured because these enzymes are involved in the major metabolic pathways of the cell.

Methods. Albino rats and mice were sacrificed by decapitation after one day of fasting and all tissues were homogenized in glass tubes (4) with glass pestles turned at 1700 R.P.M. Malic dehydrogenase was determined by the method of Potter(5). Manometer readings were taken for two 5-minute periods, following a 6-minute equilibration with a last check reading taken for a final 10-minute period. The second 5-minute period was selected for the calculation since it gave the most reproducible results. Enzymatic assays are expressed as Q_{O_2} (mm^3 oxygen/mg dry wt/hr). Succinoxidase was determined by the method of Schneider and Potter(6) which essentially is a measure of succinic dehydrogenase activity, since cytochrome oxidase is in excess in the tissues studied. After a 10-minute equilibration, readings were taken for four 10-minute periods. Enzyme assays were made on the basis of the second 10-minute period.

Results. As the data presented in Table I indicate, with the exception of liver malic dehydrogenase of mouse and rat, the activities of the tissue enzymes decrease with increase in body size, but the enzyme activity-body size ratios show considerable quantitative variation. There is no significant difference in the activity of tissue enzymes from animals of the same species even with considerable variation in body weight, as shown in Table II.

Discussion. Although the data in Table I show that the decrease in enzyme activity with body size is very significant, the lack of a logarithmic relationship suggests that con-

TABLE I. Enzyme Q_{O_2} Values for the Tissues of Cow, Rat, and Mouse.

Animal	No. of exp.	Wt, kg	Succinoxidase: Liver Q_{O_2}	Heart Q_{O_2}	Kidney Q_{O_2}	Brain Q_{O_2}
Cow	11	340	21.1 ± 4.0*	51.8 ± 7.8	69.5 ± 5.0	20.9 ± 2.0
Rat	14	.205	60.1 ± 2.8	187.0 ± 11.0	103.0 ± 5.0	42.0 ± 2.3
Mouse	12	.031	102.0 ± 4.0	221.0 ± 14.0	189.0 ± 8.0	60.5 ± 2.1
			Malic dehydrogenase:			
Cow	11	340	64.2 ± 19.1	51.6 ± 24.1	53.3 ± 13.2	20.4 ± 2.8
Rat	11	.230	104.0 ± 12.0	132.0 ± 13.0	75.0 ± 7.0	26.7 ± 3.4
Mouse	10	.030	105.0 ± 21.0	170.0 ± 54.0	91.8 ± 9.7	57.7 ± 2.0

* Stand. dev. $= \frac{Sd^2}{n-1}$.

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† Contribution No. 85 from the Department of Zoology and Entomology of the University of Tennessee.

TABLE II. Some Intra-specific Differences in the Enzymatic Activities in Mice and Rats.

Wt, g	Kidney Q_{O_2}	Heart Q_{O_2}	Liver Q_{O_2}	Brain Q_{O_2}
	Succinoxidase—Mice			
16	153	188	115	72.4
26	168	203	116	54.2
32	162	208	115	67.5
38	218	234	84.2	57.3
46	160	240	101	55.5
	Malic dehydrogenase—Rat			
230	68.4	127	121	
360	96.0	139	96.9	
	Succinoxidase—Rat			
140	86.5		52.0	40.0
172	95.7		59.6	29.9
210	108		62.5	49.5
265	93.0		54.7	34.1

trolling factors may be different from those effective in basal metabolism(1), cytochrome c(7), and cytochrome oxidase(8). The constant enzymic activity within a single species indicates indirect genetic factors regulating metabolic enzymes rather than hormonal or other direct somatic factors. That the enzymatic variation might be due partly to differences in homogenization, to deleterious effects of freezing (cow), or to dietary differences in the different species appears improbable under the experimental conditions.

Summary. Heart, kidney, liver, and brain of the cow, rat, and mouse were assayed for maximal activity of succinoxidase and malic dehydrogenase. The levels of the two enzymes generally reflect the inverse relationship of metabolism with body size in that the tissues of the cow were lowest, those of the rat intermediate, and those of mouse highest in activity for these enzymes. The enzyme levels did not vary as an exact power function of body weight, nor were there any intra-specific changes in enzyme levels with changes in body size.

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Hemolytic Action of Water Soluble Compounds Related to Mephenesin. (20170)

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The description of the muscle-relaxing and paralyzing action of mephenesin(1) led to extensive trials of the drug as a substitute for curare during anesthesia(2). The use of concentrated solutions of mephenesin by the intravenous route was, however, soon abandoned because of the frequent occurrence of intravascular hemolysis, hematuria and anuria (3). During the past few years a number of compounds structurally related to mephenesin have been discovered that have higher water solubility than mephenesin and because of this appear more suitable for intravenous administration. The present paper describes the hemolytic action of several of these compounds and compares this property with the paralyzing and lethal action of these drugs.

Methods. Determination of water solubility was made by spectrophotometric measurement of saturated solutions of each of the compounds at the wave length corresponding to maximum absorption, using the specific absorbency previously determined for each of the compounds. The partition ratio of the compounds between water and oil ($K_{w|o}$) was likewise measured spectrophotometrically using cottonseed oil, U.S.P. as the oil phase. The partition ratio of the non-aromatic ether No. 13 was obtained by measurement of the refractive index of the aqueous solutions.

TABLE I. Physical and Biological Properties of Glycerol Ethers.

Compound No.	Compound	M.p. or B.p. (mm), °C	Water sol'y 26°C, %	$K_{w/o}$, 26°C	PD_{50}* i.p., mg/kg	LD_{50}† i.p., mg/kg	Time for M/10 sol. to produce 75% hemolysis, min.	% conc. causing 75% hemolysis in 100 min.
1	3-phenoxy-1,2-propanediol	64–66	7.0	6.1	490 ± 26	1400 ±125	26	1.18
2	3-*o*-toloxy-1,2-propanediol	70–71	1.2	1.3	180 ± 18	560 ± 36	0.2	.46
3	3-*o*-methoxyphenoxy-1,2-propanediol	81–82	7.0	13.4	320 ± 23	1200 ± 67	52	1.69
4	3-*o*-ethoxyphenoxy-1,2-propanediol	66–67	>50	4.9	235 ± 15	730 ± 42	3.5	.99
5	3-(2,6-dimethoxyphenoxy) 1,2-propanediol	52–53	>50	10.1	370 ± 24	1200 ± 67	52	1.84
6	3-benzyloxy-1,2-propanediol	109–111 (0.1)	∞	3.6	660 ± 69	1650 ± 70	42	1.31
7	3-*p*-methylbenzyloxy-1,2-propanediol	57–58	10	4.9	360 ± 21	730 ± 40	1.1	.89
8	3-*o*-methoxybenzyloxy-1,2-propanediol	145–148 (0.3)	∞	8.1	340 ± 18	1150 ± 72	25	1.28
9	3-(β-phenylethoxy)-1,2-propanediol	156–157 (2.0)	∞	6.1	380 ± 33	830 ± 55	7	.92
10	3-(α-phenylethoxy) 1,2-propanediol	130–133 (0.5)	∞	3.8	350 ± 38	765 ± 36	11	1.03
11	2-phenoxy-1,3-propanediol	68–69	>50	36.0	760 ± 50	1860 ±123	130	1.82
12	2-*m*-toloxy-1,3-propanediol	66–67	9	4.0	365 ± 19	1050 ± 35	8	.97
13	2-*o*-methoxyphenoxy-1,3-propanediol	64–66	>50	5.3	800 ± 47	1710 ± 83	50	1.69
14	2-*o*-ethoxyphenoxy-1,3-propanediol	157–160 (2.0)	3.8	2.1	340 ± 16	1000 ± 84	1.5	.77
15	2-benzyloxy-1,3-propanediol	39–40	>50	36.0	650 ± 55	2300 ±207	110	1.88
16	1-ethoxy-3-isopropoxy-2-propanol	105–107	∞	2.5	765 ± 43	2480 ±141	1200	4.14

* PD_{50} median paralyzing dose in mice. † LD_{50} median lethal dose in mice.

The *paralyzing action* was tested in white mice of the CF-1 strain weighing 18-22 g. Groups of 10-20 mice were injected intraperitoneally at dose levels increasing in geometric progression by a factor of approximately 1.5. An animal was considered paralyzed when it lost the righting reflex for at least one minute. The dose producing paralysis in one-half of the animals was taken as a measure of muscle relaxing and paralyzing activity. The mortality occurring 7 days after administration of the compounds was used in calculating the LD_{50} dose. Hemolytic activity was determined according to a modification of Glassman's technic(4). The compounds were dissolved in 0.9% sodium chloride solution containing 0.015 M phosphate buffer adjusted to pH 7.4. Freshly washed rabbit red cells were added to various dilutions of each compound and the time required to achieve 75% hemolysis as determined by visual comparison with the prepared standard was taken as the end point.

Results. Certain physical and biological properties of the compounds are summarized in Table I. The high water solubility of certain compounds was rather unexpected. Thus compound No. 4, the 3-*o*-ethoxyphenoxy-1,2-propanediol, is considerably more soluble than the methoxy analogue, while the corresponding propoxy compound (not included in the table) has a solubility of less than 0.5%. It is of interest to note that the 2-*o*-ethoxyphenoxy-1,3-propanediol (No. 14) does not share the high solubility of its isomer. The 2-*m*-tolyl-1,3-propanediol (No. 12) differs from its isomers in possessing high water solubility.

There is considerable variation in the values of the partition ratios of the various compounds and there appears to be little relationship between this factor and the paralyzing activity of the compounds. Thus mephenesin (No. 2), the most active paralyzing agent of the series had a K value which does not differ too greatly from that of compound No. 16, the least active paralyzing agent. Compound No. 4 which is almost as potent a paralysant as mephenesin has a K value more than 3 times greater than mephenesin. An even more striking instance of the lack of correlation between the value of the partition ratio and central depressant activity is obtained by comparing the two isomeric benzyl ethers (No. 6 and 15). Both these compounds possess similar paralyzing activity although one has a K value 10 times greater than the other.

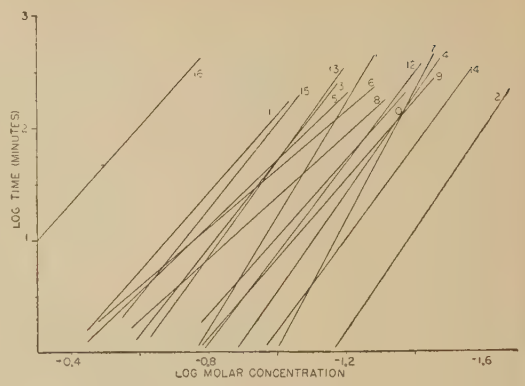

FIG. 1. Hemolytic action of certain glycerol ethers shown by plotting the logarithm of time required for 75% hemolysis against the logarithm of the molar concentration of the compounds. The curve numbers refer to the compound numbers in Table I.

The hemolytic action of the compounds is illustrated in Fig. 1 in which the logarithm of the time required for 75% hemolysis is plotted against the logarithm of the molar concentration of the compounds. The dosage response curves for the various compounds do not differ too greatly in their slopes. This may suggest a similar mechanism of hemolysis. It appears likely that the differences in hemolytic action between these compounds are quantitative rather than qualitative. Table I gives 2 parameters that can be used as a basis of comparison. One is the concentration in grams per 100 ml causing 75% hemolysis in 100 minutes. The other is the time in minutes required for a M/10 solution to produce 75% hemolysis.

The relationship between paralyzing activity and hemolytic action is of interest. Mephenesin, the strongest paralyzing agent, also has the strongest hemolytic action, while compound No. 16 which is the weakest paralysant also possesses least hemolytic action. The other compounds studied fall between these two extremes.

There appears to be a general relationship between hemolytic action and toxicity. Mephenesin exhibits greatest hemolytic action and toxicity while compound No. 16 is least

noxious in both respects. The remaining compounds show fair correspondence between toxicity and hemolytic value.

Discussion. To produce muscular relaxation during anesthesia a centrally acting relaxant such as mephenesin has certain advantages over peripherally acting relaxants because it permits relaxation without depressing respiration(5). Although mephenesin proved an effective drug for the production of muscular relaxation during anesthesia(6) the intravenous use of the drug was almost completely abandoned because of the occurrence of intravascular hemolysis. It was shown that the hemolytic action of mephenesin is independent of the total amount injected but depends on the concentration of the solution used, the presence of other hemolytic agents used as solvents and the rate of injection(7). Several of the water-soluble compounds reported in this paper have been previously described(8) but data concerning their hemolytic power have not been published. Ginzel(9) investigated the hemolytic action and pharmacological properties of *o*-methoxy, *o*-ethoxy and *o*-propoxyphenyl glycerol ethers and recommended the methoxy compound for clinical use because of its low hemolytic action. The present paper confirms the low hemolytic action of this compound. The 3-(2,6-dimethoxyphenoxy)-1,2-propanediol originally prepared by Meltzer and Doczi(10) and considered unsatisfactory by them, appears equally effective and non-hemolytic as the *o*-methoxy compound. Lambooy(11) prepared the very soluble *o*-fluoro analogue of mephenesin but did not evaluate its hemolytic action. Hine, *et al.*(12) found that 1-ethoxy-3-isopropoxy-2-propanol was very water-soluble and recommended the further study of the compound. The present paper shows that the hemolytic action of this substance is very low making the compound in this respect superior to all others tested. The very weak paralyzing action of the compound may make its clinical use impractical.

To administer mephenesin safely by the intravenous route it is desirable to use a solution not stronger than 1%. The average effective dose of mephenesin for relaxation during anesthesia is 1 g, necessitating the infusion of 100 ml of liquid. If it is assumed that the molar activity of the compounds in man is proportional to that observed in mice it would be necessary to inject 1.62 g of compound No. 3 to obtain a similar effect as observed after 1 g of mephenesin. The safe concentration of the solution would be 3.66% so that only about 44 ml of this solution would be required. To obtain equipotent effects the injection of 51.5 ml of a 2.15% solution of compound No. 4 or 52.5 ml of a 9% solution of No. 16 would be necessary. Since the detoxification mechanisms are probably taxed less after administration of the more potent compound the use of compound No. 4 would be preferable to compound No. 16.

Summary. The hemolytic action of 16 water-soluble ethers of glycerol has been examined and compared with their paralyzing and toxic action. Mephenesin was the most potent paralyzing agent and also possessed strongest hemolytic action. The 3-*o*-ethoxy-1,2-propanediol which approached mephenesin in paralyzing action, had only about one-half of its hemolytic activity. Several other water-soluble compounds which have considerably weaker hemolytic activity have been found. These compounds, however, were also much poorer paralyzing agents than mephenesin. No significant correlation between the solubility of the compounds, their water-oil partition ratios, and their paralyzing and hemolytic action was evident.

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Development of Resistance to 2,4-Diamino-5(3'4'-Dichlorphenyl)-6-Methylpyrimidine (SK5265) by *Streptococcus faecalis*.* (20171)

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The discovery by Hitchings *et al.*(1) of the inhibitory effect of a series of 2,4-diamino pyrimidines on *L. casei* led to their screening for anti-tumor effects in experimental animals. Clarke *et al.*(2) first noted the inhibitory action of these compounds on Sarcoma 180 in mice, and since that time they have also been shown to have an effect on various strains of transmitted mouse leukemia(3). Certain of these compounds have potent antimalarial activity(4) and cause many of the symptoms of antifolic toxicity in experimental animals(3, 5). The administration of 2,4-diamino-5-(3'4'-dichlorphenyl)-6-methylpyrimidine (SK 5265) to both dogs(6) and patients(7) causes a megaloblastosis in the bone marrow. With these compounds remissions are achieved in a certain percentage of children with acute leukemia(7). Since acute leukemia eventually develops resistance to all forms of therapy now known it was felt worthwhile to study the development of resistance to this new agent. In order to provide another system for the study of resistance to SK 5265, attempts were made to develop an SK 5265-fast strain of *Streptococcus faecalis* (ATCC 8043), an organism ordinarily requiring folic acid and very sensitive to the inhibitory action of SK 5265(8).

Method. Streptococcus faecalis (ATCC 8043) was grown for 18 hours at 37°C on Difco Folic Acid Assay broth in the presence of 1 mγ/ml of pteroylglutamic acid (PGA). When inoculated into similar broth containing the same amount of PGA and concentrations of SK 5265 varying from 3-300 mγ/ml, there was not half maximum growth in any of the tubes, but there was slight growth just visible after 48 hours in tubes containing 3, 10, 30 and 100 mγ/ml. Subcultures from the tube with the highest concentration of the drug were made into another set of tubes containing varying concentrations of the compound by a technic somewhat similar to those used to develop resistance to amethopterin in *S. faecalis*(9) and *Leuconostoc citrovorum* (10).

Results. A culture from the tube containing 100 mγ/ml of SK 5265 was inoculated into tubes containing 30, 100 and 300 mγ/ml, but even after 96 hours no growth occurred in concentrations above 30 mγ/ml. On the third transplant generation, however, growth occurred at 100 mγ/ml and on the fourth at 300 mγ/ml. As can be seen from Fig. 1, resistance to SK 5265 appeared very gradually and in a step-wise fashion. On the fourth transfer some growth occurred at 300 mγ/ml but from there to the 12th transfer no further increase in resistance was achieved. By the 16th transfer some growth occurred at 3 γ/ml. After a total of 54 transfers, growth occurred in 20-24 hours in liquid medium containing 1 mγ/ml of PGA and 100 γ/ml of SK 5265, and the organism is now being maintained on such a medium. In more quantitative experiments half maximum growth (HMG) occurred in the presence of 45 γ/ml of SK 5265 whereas with the parent organism HMG had occurred at 0.2 mγ/ml. No morphologic differences between the sensitive and resistant variants could be detected in Gram stained preparations. The resistant organism, *S. faecalis*/D, however, had lost its requirement for added PGA and grew on Difco Folic Acid Assay Medium without supplementary PGA in the presence or absence of SK 5265. This phenomenon has been confirmed in a second experiment in which resistance to SK 5265 was again induced in the parent strain of *S.*

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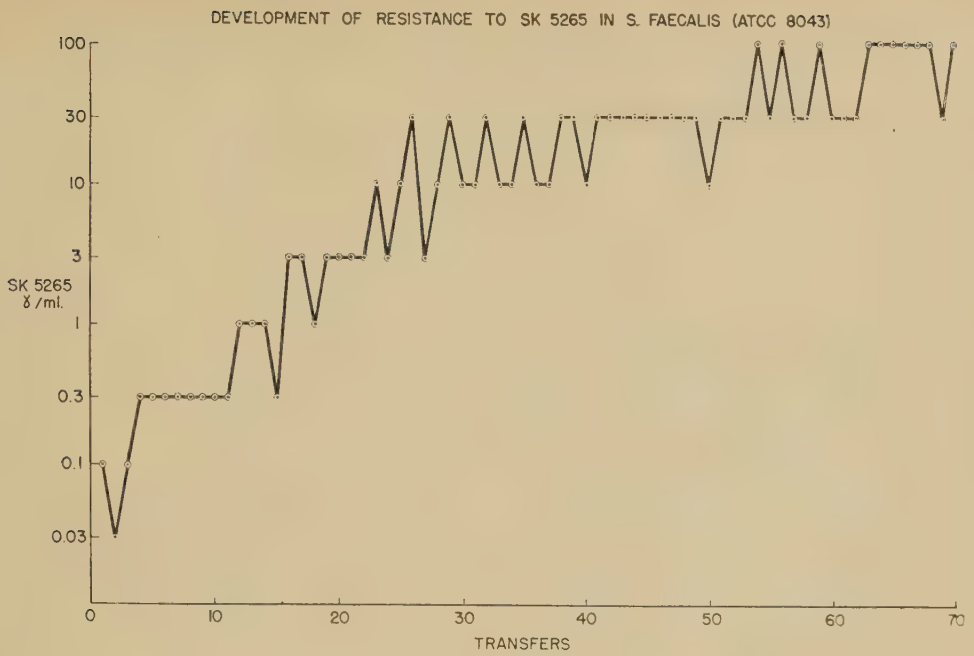

FIG. 1.

faecalis. After 13 passages in increasing concentrations of SK 5265 the organism was resistant to 3 γ/ml SK 5265 and would also grow on a Difco Folic Acid Assay Medium with no PGA or SK 5265 added.

When the parent strain of *S. faecalis* (ATCC 8043), an amethopterin resistant strain *S. faecalis*/A, (maintained in a medium containing 100 γ/ml amethopterin and no PGA), and the SK 5265 resistant strain *S. faecalis*/D, were studied for their sensitivity to amethopterin and SK 5265, by the technic of half maximum inhibition (HMI) of growth, the results presented in Table I were obtained. From this it can be seen that in the strain resistant to amethopterin there was an increase in resistance to this drug some 3 million fold, and a cross-resistance to SK 5265, approximately 250 fold. When the organism that had been made resistant to SK 5265 was compared to the parent strain, however, it was noted that there was a 225,000 fold increase in resistance to SK 5265 and a cross-resistance to amethopterin of approximately 10,000 fold.

Discussion. The development of resistance to SK 5265 by *S. faecalis* is analogous to the resistance developed to amethopterin(9). The amethopterin resistant strain (*S. faecalis*/A) maintains the same requirement for CF but a somewhat smaller requirement for PGA (11). This requirement can also be met by many of the compounds that for the parent strain behave as antagonists of PGA(9,11). Although it is impossible to state with certainty at the present time whether this is due to the utilization for growth of these compounds or to a slight contamination of these compounds with PGA or related growth promoting substances, the latter possibility seems more likely. Resting cells of this strain produce 85-250 times as much CF from a given quantity of PGA as do resting cells of the parent sensitive strain of *S. faecalis*(12). The strain of *Leuconostoc citrovorum* made resistant to amethopterin(10), however, maintained the same CF requirement as the sensitive parent strain, and was unable to utilize PGA any more efficiently than the parent strain. Even though a 2000 fold increase in resistance to amethopterin was achieved, the cross-resistance to SK 5265 increased only 10 fold(10). The SK 5265 resistant strain (*S. faecalis*/D), however after 13 generations of exposure to SK 5265 in the presence of PGA developed the ability to grow on Difco

TABLE I. Effect of Amethopterin and SK 5265 on 3 Strains of *S. faecalis*.*

	mγ/ml	
	Amethopterin HMI	SK 5265 HMI
S. faecalis 8043	.1	.2
S. faecalis/A	300000	50
S. faecalis/D	1000	45000

* All media contained PGA at 1 mγ/ml.

Folic Acid Assay medium without either PGA or SK 5265. It also has been observed that *S. faecalis*/D will grow in 18 hours to half maximum growth or more on certain other PGA deficient media in the absence of added PGA. On these same media *S. faecalis* will not grow at all in the 18 hour incubation period. It has been suggested previously in *S. faecalis*(8) and in mice(3) that SK 5265 acts at a higher level than amethopterin in the folic acid citrovorum factor metabolic pathway.

Preliminary experiments indicate that *S. faecalis*/D has become very efficient in the formation of CF from PGA. This alteration along with the greatly decreased PGA requirement indicates that the development of resistance to SK 5265 has followed the same pattern as the amethopterin resistance in *S. faecalis*. This resistance, however, appears to be more outstanding in one part, *i.e.*, the greater decrease in the PGA requirement of this culture. Biochemical studies are underway at the present time to further elucidate these mechanisms.

Summary. 1. By growing *S. faecalis* (ATCC 8043) in a liquid medium containing successively higher concentrations of 2,4-diamino-5 (3′4′-dichlorphenyl)-6-methylpyrimidine (SK 5265), a thousand fold increase in resistance as evidenced by complete inhibition of growth and a more than two hundred thousand fold increase in resistance as demonstrated by the half maximum inhibition technic has been achieved. 2. No morphologic differences between the sensitive *S. faecalis* (8043) and the resistant variant could be detected. 3. The resistant organism has lost its requirement for supplementary PGA in Difco Folic Acid Assay Medium and certain other PGA deficient media.

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Toxicity, Infectivity and Antigenicity of a Streptomycin Dependent Mutant of *Brucella abortus* (Strain 19). (20172)

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Streptomycin-resistant *Neisseriae* and *Escherichiae* were found to be non-pathogenic for mice unless the animals were treated with the drug(1,2). It was therefore to be expected that strictly dependent mutants should be applicable for immunization purposes in untreated animals. However, *Brucellae* strictly dependent on streptomycin have not yet been described, although some strains grow more abundantly in its presence(3). The following experiments were carried out with a strictly dependent mutant of *Br. abortus* which required for its optimal growth a concentration of 0.01% of streptomycin.

Origin of the strain. In the course of studies on the number of resistant mutants in cultures of *Br. abortus*(4) several broth cultures derived from single colonies were exposed to a 0.01% concentration of streptomycin. In one culture some mutants which survived after an exposure to 0.01% of the antibiotic grew better at this concentration than at 0.001% and 0.0001%. They were continuously subcultured on broth containing 0.01% of streptomycin, lost gradually their ability to grow at lower concentrations of the antibiotic and became finally dependent upon it.

Toxicity. The strains to be examined were grown on trypticase-soy-agar and washed off with small amounts of saline. Then cooled acetone was added, the dead cells were removed by centrifugation, resuspended in acetone, centrifuged again, dried *in vacuo* and prepared as 1% suspensions in saline. One half of each suspension was left unheated, while the other half was heated at 100°C for 2 hours. Corresponding groups of 10 mice received 12, 10, 8, 6 and 4 mg of unheated and heated bacteria intraabdominally and were held under observation for 14 days. The LD50 was 9.2 mg for the streptomycin-dependent, 9.6 mg for the streptomycin-resistant and 7.3 mg for the non-resistant *Br. abortus* 19 parent strain. No differences were observed between toxicity of unheated and heated bacteria.

Infectivity. Fresh agar cultures were washed off with broth and suspensions were prepared which contained 3.2 x 10^9 *Brucellae* per cc. Suitable dilutions of this suspension were poured into plates and the dose to be injected checked by plate counts. Groups of 20-25 mice received intraabdominally varying quantities of *Brucellae* suspended in 0.5 cc of broth. After suitable time intervals groups of five mice were killed by ether, the organs removed aseptically, ground with glass powder in porcelain mortars and the homogenous suspensions of fine particles were taken up in 50 cc of streptomycin broth. From these suspensions series of tenfold dilutions were made and 0.2 cc of suitable dilutions plated. The average number of *Brucellae* per organ was determined by plate counting. Table I shows the bacterial counts in the organs of mice infected with the non-resistant parent strain (NR) and the streptomycin-dependent mutant (D). When 3 x 10^6 NR-bacteria were injected, concentrations of more than 10^6 bacteria per spleen were observed and the bacteria persisted after 60 days. When the same quantity or 10 and 100 fold quantities of D-bacteria were injected, no such concentrations were ever observed, and the bacteria disappeared within 45 days from the spleen. When a 1000-fold infective dose of D-bacteria was given, more than 10^6 bacteria per spleen were counted, but they disappeared within 60 days. The administration of streptomycin did not prevent the final disappearance of the D-bacteria from spleen and liver. The spleen enlargements produced by D-bacteria were relatively small, and 5 x 10^7 of them were needed in order to produce enlargements for which about 5 x 10^3 NR-bacteria were sufficient as shown in Table II.

Agglutinogenic power. Twelve rabbits were divided into 4 groups and received at 3-day

TABLE I. Number of *Brucellae* in the Organs of White Mice Infected with Strains NR (Non-Resistant against Streptomycin) and D (Streptomycin-Dependent).

Organ	Strain	No. of bacteria inj.	No. of *Brucellae* per organ at intervals (days) after onset of infection 8	14	30	45	60
Spleen	NR	3×10^6	3×10^6	2×10^6	1×10^5	2×10^5	6×10^4
	D	2×10^9		5×10^6	5×10^2	1×10^2	0
		5×10^8		2×10^5	8×10^2	0	0
		5×10^7		7×10^4	3×10^2	0	0
		5×10^6	8×10^4	8×10^3	2×10^1	0	0
		5×10^6*	9×10^3	6×10^3	1×10^2	0	0
		5×10^6†		2×10^4	1×10^3	0	0
Liver	NR	3×10^6	4×10^5	2×10^4	1×10^4	5×10^3	8×10^2
	D	2×10^9		4×10^3	1×10^2	0	0
		5×10^8		2×10^4	2×10^2	0	0
		5×10^7		2×10^3	2×10^2	0	0
		5×10^6	2×10^3	8×10^2	0	0	0
		5×10^6*	3×10^3	0	0	0	0
		5×10^6†		2×10^3	2×10^1	0	0

* Received daily 1 mg streptomycin 7 days following onset of infection.
† " " 1 mg " 14 " " " " " .

TABLE II. Average of Relative Spleen Weight, Expressed as % of Body Weight, in Group of Infected Mice. Normal value 0.6.

Strain	Time after inj. (days)	Relative spleen wt after inj. of No. of *Brucellae* 5×10^8	5×10^7	5×10^6	5×10^4	5×10^3	5×10^2
NR	8			.8	1.0	.7	.5
	15			1.4	1.1	1.0	.6
	30			1.1	1.2	1.0	.7
D	8	.8	.9	.6	.5		
	15	.9	1.1	.6	.6		
	30	.9	.6	.7	.5		

intervals intravenously 1.0, 2.0 and 3.0 cc of suspensions containing 10^9 D- or NR-bacteria per cc, both living and killed by acetone. At different time intervals after the last injection blood was taken and the agglutinin titers determined. Table III shows that the average agglutinin titer in the groups immunized with dead bacteria decreased quickly and reached the value of 1:200 after 45 days. No differences of agglutinogenic power were observed between dead NR- and D-bacteria. On the other hand, the agglutinins persisted for a longer period after the administration of living bacteria. Although the initial titers were nearly equal for both living NR- and D-bacteria, they decreased more quickly when living D-bacteria were employed.

Guinea pigs received 3 injections of 3 x 10^9 bacteria at 10-day intervals. Thirty days after the last injection, titers of 1:1000 were observed in sera of the animals treated with living NR-bacteria; 1:500 in those which were treated with living D-bacteria, while in the groups treated with acetone-killed bacteria the titers did not rise over 1:200.

Immunization experiments. Groups of 20 mice received intraabdominally varying quantities of living D- and killed D- and NR-bacteria. Three injections were given at 7-day intervals. 30 days after the last injection, all immunized mice and a group of 20 untreated mice received 10^6 *Brucellae* (*Br. abortus* 2308) intraabdominally. Five mice were killed at different periods after the challenge infection and the average number of *Brucellae* per spleen determined. Bacterial counts on plain agar and streptomycin-agar showed how many organisms of the immunizing D- and the non-resistant challenge strain were present. Table IV shows that in the group immunized with 1.5 x 10^{10} living D-bacteria the NR-bacteria did not appear in the spleen so long as D-bacteria were still present. In the groups treated with killed bacteria the number of the NR-challenge bacteria was markedly reduced as compared with their number in

TABLE III. Average Agglutinin Titers in Serums of Rabbits Immunized with Living and Acetone-Killed D- and NR-Bacteria.

Bacteria inj.	Titers observed in sera at different intervals (days) after last inj., ×1000					
	7	14	30	45	60	90
NR, living	1: 90	1:110	1:70	1:5	1:2	1:1
D, living	1:100	1:120	1:50	1:2	1:1	1:0.5
D & NR, killed by acetone	1: 40	1: 22	1: 5	1:0.2	1:0.2	1:0.1

TABLE IV. Immunizing Effect of 3 Injections of Living and Acetone-Killed D- and Acetone-Killed NR-Bacteria.

Antigen inj.	No./inj.	No. of *Brucellae*/spleen (D and NR) at different intervals (days) after inj. of challenge dose				
		7		15		30
		D	NR	D	NR	NR
D living	1.5×10^{10}	2×10^2	0	0	3×10^2	0
	$\times 10^8$	3×10^2	3×10^2	0	3×10^2	0
	$\times 10^6$	0	2×10^3	0	4×10^3	6×10^2
D killed	$\times 10^{10}$	0	3×10^2	0	2×10^3	4×10^2
	$\times 10^8$	0	2×10^3	0	2×10^4	7×10^3
	$\times 10^6$	0	2×10^3	0	2×10^4	3×10^4
NR killed	$\times 10^{10}$	0	3×10^2	0	3×10^2	9×10^4
	$\times 10^8$	0	8×10^2	0	2×10^3	7×10^4
	$\times 10^6$	0	1×10^4	0	7×10^3	4×10^4
Controls untreated		0	3×10^6	0	4×10^4	1×10^7

the untreated animals. The number of the NR-challenge bacteria was lower in the spleen of mice immunized with living D-bacteria than in those immunized with dead D- or NR-bacteria. Living NR-bacteria were not employed for the immunization since by the method employed it was impossible to determine whether the bacteria isolated after the challenge injection belonged to the immunizing or to the challenge strain.

Stability of the D-mutant. The D-strain was subcultured 6 times at monthly intervals on agar slants containing 0.01% of streptomycin and the appearance of non-dependent mutants tested for by inoculation of 10^{10} bacteria on plain agar and broth. No growth appeared without the presence of streptomycin.

Summary. A streptomycin-dependent mutant (D) of *B. abortus* (strain 19) showed the following properties: 1. Its toxicity for mice was almost equal to that of the streptomycin-resistant (R) and non-resistant (NR) parent strains. It produced infections of limited duration with relatively low concentrations of microorganisms in the spleens of white mice, and disappeared from the spleen within 45 to 60 days. Spleen enlargements were observed when 5 x 10^7 or more microorganisms were injected. Subcutaneous injection in man did not result in serious reactions. 2. The agglutinogenic power of the acetone killed D-bacteria in rabbits and guinea pigs was equal to that of NR-bacteria. As living antigens D- and NR-bacteria produced high initial titers, but those produced by the D-bacteria decreased more quickly. Three injections of living D-bacteria prevented completely the appearance of virulent NR-bacteria given as challenge dose in the spleen or lowered their number markedly as compared with the non-immunized controls. Killed vaccines prepared from NR- and D-strains did not exert such marked effects.

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Distribution and Persistence of Aureomycin in the Chick Embryo.* (20173)

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In connection with our investigation of the effect of aureomycin on meningopneumonitis virus in chick embryos(1), it was felt desirable to determine the distribution and persistence of the antibiotic in this host following inoculation into the allantoic cavity. This report summarizes the findings obtained in these determinations.

Materials and methods. Chick embryos were inoculated with 1 mg of crystalline aureomycin by the allantoic route, and at various intervals thereafter tissues, allantoic fluids and plasma were harvested. For assay of the drug content in *allantoic fluids,* a sample of a pool of fluids from 3 eggs was used. *Allantoic membranes* were usually removed from the same eggs from which fluid samples had been taken, washed 6 to 7 times in broth-buffered water mixture and triturated in a Ten Broeck grinder. The resulting suspension could be removed from the grinder with a pipette without the addition of diluent. *Organs* were ground in a mortar with saline. *Plasma* was obtained by collecting blood from 12- to 13-day embryos (younger embryos were found to be unsatisfactory for bleeding with syringe and needle) in an equal volume of 2% sodium citrate. The bleeding of the embryos was done by making a window in the shell over a large vessel of the allantoic membrane. Sterile mineral oil was used to make the shell membrane transparent, and blood could be drawn from the vessel without entering the allantoic cavity. All preparations were frozen immediately and kept at –20 to –25°C until the assays were done. At the time of assay an equal volume of saline was added to the suspensions of membranes and organs and extraction of aureomycin was allowed to proceed at room temperature for 30 to 60 minutes. The assay was done on the saline extract. All figures are presented as γ per ml. Conversion to γ per g was not warranted because the values are not absolute values but rather approximations (complete extraction was not possible). The *zero hour* interval in Table I actually involved 2 to 5 minutes to carry out the harvesting procedure. Each egg at this interval was inoculated and harvested immediately. This was true of the embryos which were bled also; the shell windows were prepared beforehand. The method of assay, that of Dornbush and Pelcak(2), was sensitive to $\pm$ one 2-fold dilution step.

Experimental. It can be seen in Table I that the amount of drug present in the allantoic fluid immediately after inoculation (0 hour) was only slightly less than the expected theoretical amount based on dilution of one mg of drug in the allantoic fluid of the egg. Though there appeared to be some variation in the actual values (2-fold variations are considered within limits of experimental error), the general picture was one of a progressive decrease in the amount of the drug with time. The difference between concentrations of aureomycin at time zero in the fluids of 9-day and 11-day embryos can be explained by the larger amount of allantoic fluid in the 11-day eggs. It is interesting, however, that the rate of loss was much greater in the 11-day-old embryos. The difference is especially significant in view of the fact that the increase in amount of allantoic fluid and hence progressive dilution of the drug in the course of growth is greater in the embryos which are 9 days at time of treatment, than in those which are 11 days old.

The changes in the amount of drug associated with the allantoic membrane in relation to time seemed to be similar to those observed with fluids but on a much lower scale. As mentioned under *materials and methods,* the figures in Table I for the amount of drug per ml of membrane or organ do not represent

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TABLE I. Concentration of Aureomycin in Embryo Tissues.*

Time (hr)	Allantoic fluid† (γ/ml) 9 day	11 day	Allantoic membrane† (γ/ml) 9 day	11 day	Plasma‡ (γ/ml) 11 day			Organs (γ/ml) Embryo	Liver 11 day	Brain 11 day	Heart 11 day
0	150	75			6.2	.2	6.2				
1	125		5.2				0				
2		45		1.7	1.5	.1	12.5	0	.2	0	0
3	50						12.5				
4				1.0	12.5	<.1	0				
6	150	20	4.0	1.7		<.1	1.5	1.5	.2	0	0
8	100	12				<.1					
20		7			0						
25	50	7	6.3	1.5	0	<.1		0.37	.2	0	0
48	40	2	7.0	.5				0	0	0	0
72	12		4.0								
96		2		.37				0	0	0	0
120	7		1.5								
144		0.75		.2					0	0	0

Sensitivity varied from 0.05 to 0.1 γ/ml.

* In most instances, crystalline aureomycin + glycinate buffer was used (unbuffered crystalline aureomycin was used in two instances with essentially the same results).

† Determinations on fluids and membranes were carried out in several experiments, and most figures represent the average of two or three results.

‡ Because of irregularities, three individual experiments (corrected for dilution factor) are shown.

the exact amount of the drug which is associated with the tissue. However, it can be seen that a continuous decrease in amount of aureomycin occurred with the increase in time.

The results of assays on plasma appear somewhat irregular. It would seem, however, that drug was present in the blood stream but for a short time.

Of the organs tested, the liver was the only one which showed any detectable amount of aureomycin. This was an exceedingly small quantity. No organs other than the brain, heart and liver were harvested because of the difficulty in distinguishing them in these young embryos.

Summary. Data were presented on the distribution and persistence of aureomycin in the allantoic fluids and tissues of 9- and 11-day-old embryos following injection of the drug into allantoic cavity. The drug was found associated with allantoic fluid, membrane, blood and liver, but not with brain or heart. The amount of drug found following injection decreased with an increase in time.

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Influence of Oxygen Tension on Tetrazolium Reduction by Epiphyseal Cartilage of Rachitic Rats *in vitro*.* (20174)

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Little is known concerning oxidative processes in cartilage. Oxygen uptake has been demonstrated (1-3) but in amounts so small as to be comparable only with the oxygen consumption of mammalian erythrocytes. Articular cartilage of the horse and of the rabbit has been shown to reduce methylene

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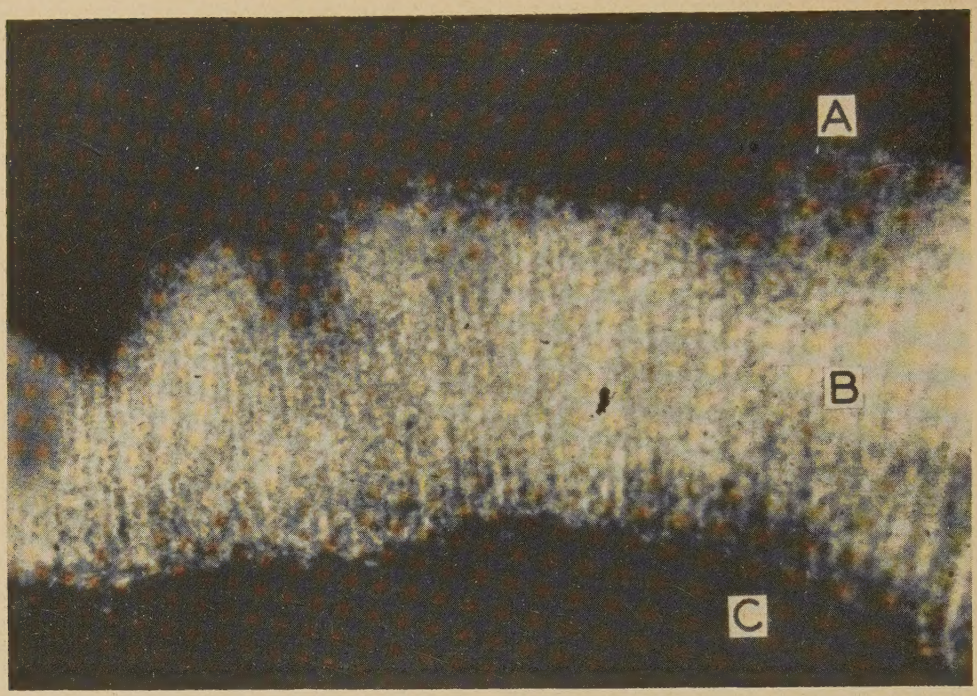

FIG. 1. Reduced triphenyltetrazolium chloride (TTC) deposition in epiphyseal cartilage cells of a rachitic rat bone slice after 1 hour incubation at 37°C in 0.5% TTC-Ringer-phosphate in an atmosphere of 100% nitrogen. A—Epiphysis. B—Epiphyseal cartilage. C—Metaphysis.

blue, with a concomitant increase in oxygen consumption(2). Only traces of cytochrome have been seen on spectroscopic examination (3) and none was demonstrable histochemically(4). Follis(5) has shown both succinic and citric dehydrogenase activity in cartilage. Calcification of rachitic rat epiphyseal cartilage slices *in vitro* has been shown to proceed equally well in both oxygen and nitrogen(6). It has been shown(7,8) that 2,3,5,-triphenyl tetrazolium chloride (TTC), a colorless, water-soluble reagent, when incubated with viable tissue slices, is connected to a deep red, water-insoluble formazan which is deposited within the cytoplasm of the cell. This process is thought to occur as a result of its reduction by intracellular dehydrogenase activity. The reduction of ditetrazolium salts by liver homogenates(9) and of TTC by liver, kidney and adrenal slices(10) has been found to take place more actively under anaerobic than aerobic conditions. This phenomenon has been interpreted as evidence of a competition between the tetrazolium salt as a hydrogen acceptor and the cytochrome-cytochrome oxidase system(9).

In the present study TTC has been incubated *in vitro* with rat epiphyseal cartilage slices under anaerobic and aerobic conditions. A profound difference has been noted in the extent of TTC reduction by cartilage cells under these conditions, comparable to that observed with other tissues.

Methods. Thirty-day-old male rats (Carworth Farms), mildly rachitic after 7 to 10 days on the Schneider-Steenbock low-phosphorus diet(11), were sacrificed by a blow on the head, and contiguous thin slices of the upper ends of the tibiae were cut free-hand. These were placed in Erlenmeyer flasks containing 10 cc of Ringer-phosphate (pH 7.4) and 0.5% TTC. Following equilibration for 5 minutes with 100% oxygen or 100% nitrogen, incubation was carried out in a closed system for one hour at 37°C with constant slow shaking in the Warburg bath. Since the colored formazan fades on exposure to light, the bone slices were examined immediately

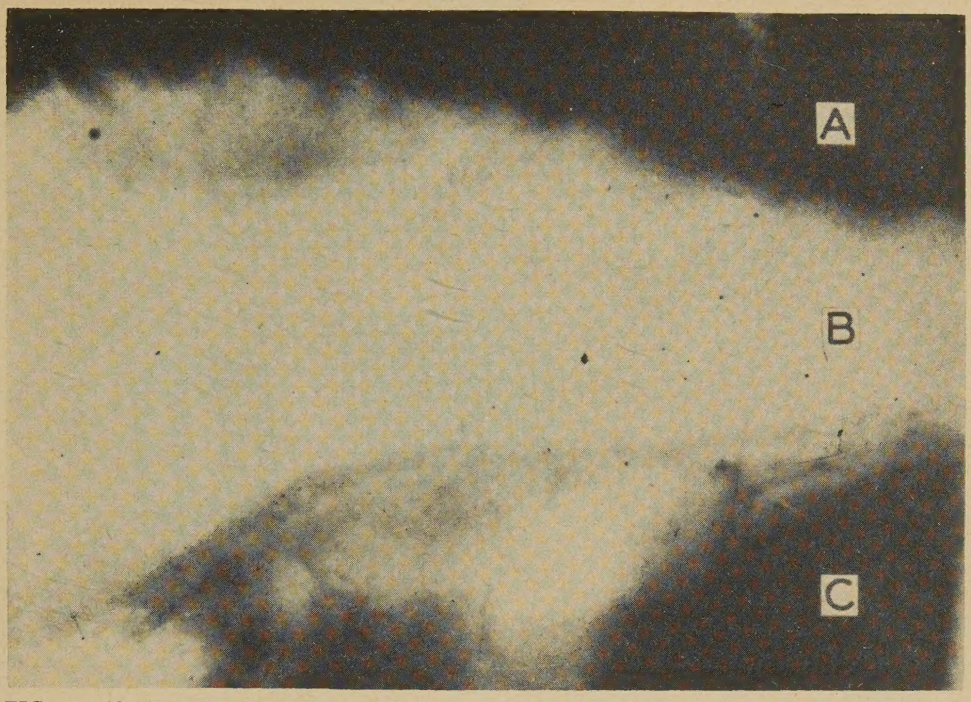

FIG. 2. Absence of reduced triphenyltetrazolium chloride (TTC) deposition in epiphyseal cartilage cells of a rachitic rat bone slice after 1 hour incubation at 37°C in 0.5% TTC-Ringer-phosphate in an atmosphere of 100% oxygen. A—Epiphysis. B—Epiphyseal cartilage. C—Metaphysis.

after incubation under 80x magnification for evidence of formazan deposition and photographed to obtain a permanent record.

Results. In slices equilibrated with an atmosphere of nitrogen during incubation a regular pattern of formazan deposition could be seen within the cartilage cells throughout the epiphyseal cartilage plate (Fig. 1). In contrast, there was virtually no deposition of formazan in the cartilage cells exposed to an atmosphere of 100% oxygen during incubation (Fig. 2). The difference was consistently noted in all 24 experiments (Table I).

The adjacent epiphyseal and metaphyseal bone was diffusely stained by the formazan, assuming a pink color during aerobic and a deep red color during anaerobic incubation. That this formazan deposition was attributable to the activity of bone marrow elements was evident from the absence of any TTC reduction on either aerobic or anaerobic incubation of bone slices from which the marrow had been removed.

TABLE I. Reduction of TTC by Epiphyseal Cartilage in Oxygen and in Nitrogen, 24 Exp.

Formazan deposition	Gas atmosphere	
	100% O_2	100% N_2
4+	0	24
3+	0	0
2+	0	0
+	8	0
0	16	0

Summary and conclusions. 2,3,5-triphenyl tetrazolium chloride when incubated with slices of epiphyseal cartilage obtained from rachitic rats is much more intensively reduced to its formazan in nitrogen than in oxygen. The reduced formazan is deposited within the cartilage cell. Based on the current interpretation of a similar phenomenon in other tissues, this observation provides additional evidence for the existence of oxidative enzyme systems in cartilage cells.

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